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GENOMIC SIZE AND PLOIDY LEVEL PATTERNS OF INTERMOUNTAIN WEST *LEPIDIUM* DETERMINED USING FLOW CYTOMETRY

Robert W. Lichvar¹

ABSTRACT.—The taxonomic status of members of the genus *Lepidium* in the Intermountain West has been in flux for years. Species concepts and classification of these endemic species from the western United States center on the highly variable *L. montanum* complex. Until recently, classification treatments that use morphological features in this group have been adequate, but as new species are discovered and more locations reported, the limited number of morphological features available for classification and defining species concepts has led to more uncertainty about taxonomic rankings. As part of a molecular and morphological-based treatment of the group, flow cytometry was used to evaluate 58 collections of *Lepidium* from the Intermountain West. The ploidy level for 14 species and 11 varieties of *Lepidium* was surveyed to assess whether ploidy levels affected the interpretation of taxonomic rankings. Of the *Lepidium* taxa surveyed, 90% were tetraploid, and several were diploid and hexaploid. Tetraploid occurrences crossed all major species and varieties. No geographic distribution or habitat patterns were associated with ploidy level.

RESUMEN.—Durante años, el estado taxonómico de los miembros del género *Lepidium* en el área intermontañosa del oeste ha estado en constante cambio. Los conceptos de especie y la clasificación de estas especies endémicas del oeste de los Estados Unidos se enfocan en el complejo *L. montanum* sumamente variable. Hasta hace poco, los tratamientos de clasificación que utilizan características morfológicas en este grupo han sido adecuados, pero a medida que se descubren nuevas especies y se registran más ubicaciones, el limitado número de características morfológicas disponibles para la clasificación y la definición de los conceptos de especie ha dado lugar a una mayor incertidumbre sobre las clasificaciones taxonómicas. Como parte de una técnica molecular y morfológica del grupo, se utilizó citometría de flujo para evaluar 58 colecciones de *Lepidium* en el área intermontañosa del oeste. Se realizó un estudio de ploidía en 14 especies y 11 variedades de *Lepidium* para evaluar si los niveles de ploidía afectaban la interpretación de las clasificaciones taxonómicas. El 90% de los *Lepidium* estudiados resultaron ser tetraploides, junto con varios taxa que son diploides y hexaploides. Las ocurrencias tetraploides cruzaron las principales especies y variedades. No se encontraron patrones de distribución o de hábitats geográficos asociados con el nivel de ploidía.

The Brassicaceae are distributed worldwide and consist of 49 tribes, 321 genera, and more than 3660 species (Al-Shehbaz 2012). The use of chromosome numbers for distinguishing differences between genera in the Brassicaceae has been limited (Rollins 1993). Even though chromosome numbers are not taxonomically definitive in Brassicaceae, in some cases the numbers are consistent and not random among different species groups. Rollins (1955, 1979, 1993) showed that certain species groups within select genera share a similar base number and, in some cases, are associated with groups with specific morphological characteristics. As such, chromosome numbers have supported taxonomic treatments in Brassicaceae between some species (Rollins 1963, Mulligan 1976) but have not been useful in other treatments (Mulligan 1966). As a whole, chromosome counts across the family express frequent

occurrence of hybridization, apomixis, and variation in ploidy levels; therefore, chromosome counts may or may not be supportive for taxonomic purposes.

Base chromosome numbers in the family vary from $x = 4$ to $x = 17$, with more than one-third of the taxa having karyotypes of $x = 8$ (Warwick and Al-Shehbaz 2006). Chromosome counts reported by Warwick and Al-Shehbaz (2006) represent 232 of the 321 genera and 1558 of the 3660 species in Brassicaceae. Of the species reported, approximately 37% are assumed to be polyploids. The percentage estimates may be even higher if diploid species are shown to be paleopolyploids that formed from diploidization (Lysak et al. 2005). Frequent auto- and allopolyploid events complicate the utility of chromosome numbers even further, along with chromosome reshuffling, fusions, and fissions, which have caused intrageneric numeric

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variation and descending or ascending dysploidy (Lysak et al. 2005). Thus, it is impractical to use the base number for taxonomic interpretation in some genera. In the Brassicaceae, $x = 4$ is the lowest base number and is found in 2 unrelated genera—*Stenopetalum* R. Brown from Australia and *Physaria* (Nutt.) A. Gray. The highest base number is $x = 128$, found in *Cardamine* (Easterly 1963, Al-Shehbaz 1988). In *Lepidium* L., the base number is reported as $x = 8$, based on 231 counts from 60 species (Warwick and Al-Shehbaz 2006). In the reported counts for *Lepidium*, 34% were diploid, 14% both diploid and polyploid, and 52% entirely polyploid. Ploidy numbers in the genus are reported as $2n = 16, 24, 28, 32, 40, 48, 64,$ and 80.

In addition to chromosome counts for supporting the evaluation of ploidy levels, the amount of DNA per nucleus can now be determined using techniques such as flow cytometry (FC; Bennett and Leitch 2005, Doležel and Bartoš 2005). The species of Brassicaceae analyzed with FC to date have shown small nuclear DNA contents and a narrow range of variation (Bennett and Leitch 2005). Genome size can now be superimposed over gene marker-based phylogenies for assessing ancestral DNA content, and FC has provided the ability to trace genome size evolution (Marhold and Lihová 2006). Likewise, DNA content in polyploids of recent origin is expected to be proportional to the ploidy level for the purposes of assessing ploidy level (Bennett et al. 2000). This pattern is reported in several species of *Draba* L., which include diploids, triploids, tetraploids, hexaploids, and up to 16-ploids. This evidence is also supported by molecular markers (Grundt et al. 2005, Jordon-Thaden and Koch 2008).

Polyploidization and hybridization often result in a reticulate pattern of evolution (Marhold and Lihová 2006). These events make reconstruction of evolutionary relationships challenging. Reticulate evolution can be detected by incongruences between phylogenetic trees derived from plastidic (cp) and nuclear (nr) DNA. These incongruences are common in *Lepidium* between cpDNA and nrDNA ITS (internal transcribed spacers) markers, with common polyploids suggesting allopolyploid speciation in the genus (Mummenhoff et al. 2001, 2004). It has been suggested that increases or decreases in ploidy levels as determined

from genome size can be used to indicate the contributions of different parental origins (Wendel et al. 2002, Kellogg and Bennetzen 2004, Leitch and Bennett 2004).

As part of a taxonomic evaluation of native species of *Lepidium* from the Intermountain West of the United States, a series of research efforts has been undertaken. Recent taxonomic treatments mention that this group needs further molecular studies to clarify species concepts and taxonomic rankings used in the group (Holmgren 2005, Al-Shehbaz and Gaskin 2010). As part of those efforts, this survey reports on ploidy-level studies using flow cytometry methods. The morphological differences that distinguish species and infraspecific taxa within this geographic region are faint but consistent within taxa, implying possible polyploidy, reticulate evolution, or recent hybrid origins. These differences have led to uncertainty about the taxonomic arrangement of the group under review, particularly about what constitutes the species level and how these taxa are related to each other (Hitchcock 1936, 1950, Rollins 1993, Holmgren 2005, Al-Shehbaz and Gaskin 2010).

I used ploidy-level information derived from flow cytometry methods to evaluate whether ploidy-level patterns are helpful in revealing a taxonomic interpretation of Intermountain West *Lepidium*. One aspect I evaluated was whether ploidy-level patterns are found along geographic ranges associated with landscape or distribution patterns as reported in the literature (Suda et al. 2007, Whittemore and Olson 2011). In this study, 2 main questions addressed the classification of western *Lepidium*: (1) whether ploidy-level occurrences are associated with geologic, geographic, or ecological settings in the taxonomically problematic *Lepidium* and (2) whether ploidy levels are associated with certain species groups or certain infraspecific taxa within a species.

METHODS

Plant Material

Seeds were collected in the field, and plants were grown in a greenhouse to provide green leaf tissue for flow cytometry (FC; Table 1). Seeds were obtained during several extensive field trips throughout the Intermountain West between 2007 and 2009. Search locations of *Lepidium* of interest were developed using

location data from herbarium specimens at the Gray Herbarium (GH), the New York Botanical Gardens Virtual Herbarium (Thiers, continuously updated), and the Calflora (2008) herbaria databases online. Other voucher collections, including seeds, were collected by several botanists and shipped to the University of Alaska–Fairbanks (UAF; Table 1).

Seeds were collected from 3–5 individuals per population. Leaf materials were collected from the same individuals for DNA extraction, and voucher specimens were collected from the population for morphological analysis and taxonomic identification.

Geographic patterns of western *Lepidium* were evaluated by developing distribution maps of data collection points using a geographic information system (GIS). Geographic Universal Transverse Mercator (UTM) coordinates were assigned to all *Lepidium* voucher specimens and displayed as point locations in ArcGIS 10.0. Based on the species localities, a distribution map was then created and digitized.

Due to uncertainty of the taxonomic status of various rankings, the nomenclature follows several sources. These include Rollins (1993), Hitchcock (1950), Holmgren (2005), and Al-Shehbaz and Gaskin (2010).

Flow Cytometry

Live leaf materials were collected from greenhouse-grown *Lepidium* and kept on ice until processing occurred, generally within 1–3 h. Leaves were placed in a petri dish with equal amounts of leaves from the size standard (*Glycine max* (L.) Merr. ‘Polanka’) maintained in a growth chamber at UAF. Leaf material was chopped in 0.5 mL of cold chopping buffer using a stainless-steel razor blade. The chopping buffer was modified from Otto (1990) Buffer I by adding 0.5% v/v of TritonX100 rather than Tween 20. As the leaves were chopped, an additional 0.5 mL of cold chopping buffer was added. The samples were filtered through a 30-mm Partec CellTrics® filter and centrifuged for 20s at 3500 rpm. The supernatant was drawn off, and 2 mL of RNase was added to the pellet. The pellet was resuspended in 0.2 mL of propidium iodide staining buffer (28.65 g dibasic sodium phosphate, 50 mg propidium iodide, 200 mL deionized water). Samples were stained in the dark for 40 min prior to flow cytometry application.

Flow cytometry (FC) was performed on a BD Bioscience FACSaria flow cytometer (BD Bioscience, San Jose, CA) equipped with FACSDiva Software (BD Bioscience, San Jose, CA). Samples were run until 10,000 nuclei were scored. The DNA content was calculated by comparing the mean peak fluorescence with the internal reference standard, *Glycine max* (L.) Merr. ‘Polanka’ (2C = 2.5 pg DNA; Doležel and Bartoš 2005). Following Doležel and Bartoš (2005), the 2C value of each sample (somatic cell nuclear content) was estimated as the (sample peak mean/standard peak mean) × standard 2C DNA content (2.5 pg DNA). To verify the range of DNA content, C values were compared to values reported in the Kew Royal Botanical Gardens DNA C-values database (Bennett and Leitch 2012). The 2C values were used to determine diploids and tetraploids by comparison to reported polyploidy levels from published chromosome counts (Warwick and Al-Shehbaz 2006).

Ploidy Counts Reported in the Literature

Chromosome counts for 6 of the taxa surveyed here have been previously reported in the literature. These are *L. dictyotum* A. Gray (2n = 32; Mummenhoff et al. 2004), *L. latifolium* L. (2n = 24), *L. lasiocarpum* Nutt. ex Torrey & Gray (2n = 32), *L. integrifolium* Nutt. ex Torrey & Gray var. *heterophyllum* S. Wats (2n = 16), *L. montanum* Nutt. ex Torrey & Gray var. *canescens* (Thell.) C.L. Hitchc. (2n = 32), *L. montanum* var. *jonesii* (Rydb.) C.L. Hitchc. (2n = 32), and *L. montanum* var. *montanum* (2n = 16; Warwick and Al-Shehbaz 2006).

RESULTS

Fifty-eight samples of *Lepidium* individuals representing 14 species and 11 varieties were surveyed (Table 1). Of these 58 samples, 52 (90%) were tetraploids. Ploidy levels for the remaining taxa surveyed here are reported for the first time. For the other 3 taxa in this survey that were not tetraploids, the range of ploidy levels included diploid and hexaploid. The taxa having other than a tetraploid count were *L. integrifolium* (hexaploid), *L. montanum* var. *canescens* (diploid), and *L. montanum* var. *cinereum* (C.L. Hitchc.) Rollins (syn. var. *stellae* Welsh & Reveal) (hexaploid).

The distributions of ploidy levels across species surveyed are shown in Fig. 1. The

TABLE 1. *Lepidium* species, voucher numbers, collection locations, 2C values, ploidy levels, and previous literature reports.

Species	Voucher number	Location	2C	Inferred ploidy level	Previous literature reports
<i>L. albyssoides</i> A. Gray var. <i>albyssoides</i>	10502	Big Piney, WY	1.619	Tetraploid, 2n = 32	Rollins (1993), n = 16
	8443	Sweetwater Canyon, NM	1.526	Tetraploid, 2n = 32	
	8519	Dinosaur National Monument, CO	1.738	Tetraploid, 2n = 32	
	8519c	Dinosaur National Monument, CO	1.451	Tetraploid, 2n = 32	
	10624 (f/d)	Hay Gulch, CO	1.699	Tetraploid, 2n = 32	
<i>L. albyssoides</i> var. <i>eastwoodiae</i> (Wooton) Rollins	8909a	White Sands National Monument, NM	1.357	Tetraploid, 2n = 32	
	8509b	White Sands National Monument, NM	1.346	Tetraploid, 2n = 32	
<i>L. barnebyanum</i> Reveal	8909c2	White Sands National Monument, NM	1.327	Tetraploid, 2n = 32	
	10624	Hay Gulch, CO	1.444	Tetraploid, 2n = 32	
<i>L. crenatum</i> (E.L. Greene)	8520	Duchesne, UT	1.551	Tetraploid, 2n = 32	
	8518	Hamilton, CO	1.603	Tetraploid, 2n = 32	
	8518a2	Hamilton, CO	1.384	Tetraploid, 2n = 32	
	8518b3	Hamilton, CO	1.402	Tetraploid, 2n = 32	
	8525	North rim of Snake River, ID	1.506	Tetraploid, 2n = 32	
<i>L. davisi</i> Rollins	8525	North rim of Snake River, ID	1.38	Tetraploid, 2n = 32	Mummenhoff et al. (2004), 2n = 32
	8527	North of Sparks, NV	1.228	Tetraploid, 2n = 32	
<i>L. fremontii</i> S. Wats. var. <i>stipulata</i>	8528b	Buffalo Well, NV	1.229	Tetraploid, 2n = 32	
	8528	Buffalo Well, NV	1.458	Tetraploid, 2n = 32	
<i>L. huberi</i> S.L. Welsh & Goodrich	10465	Wamsutter, WY	1.75	Tetraploid, 2n = 32	
	10456	Vernal, UT	1.555	Tetraploid, 2n = 32	
<i>L. integrifolium</i> Nutt. ex Torrey & Gray	8522	Bear River Wildlife Area, WY	2.001	Tetraploid, 2n = 32	
	8522	Bear River Wildlife Area, WY	3.117	Hexaploid, 2n = 64	
<i>L. integrifolium</i> var. <i>heterophyllum</i> S. Wats.	10452	Cokeville, WY	1.307	Tetraploid, 2n = 32	
	24083	Cedar City, UT	1.239	Tetraploid, 2n = 32	
<i>L. latifolium</i> L.	8534	Ely, NV	0.892	Diploid, 2n = 16	Mulligan (1957), 2n = 24; Queirós (1973), 2n = 24; For others, see Warwick and Al-Shehbaz (2006)
	8534	Ely, NV	0.768	Diploid, 2n = 16	Queirós (1979), 2n = 48
<i>L. montanum</i> var. <i>canescens</i>	8530	Battle Mountain, UT	1.587	Tetraploid, 2n = 32	Rollins and Rüdtenberg (1971), 2n = 32
	8529	Paradise Valley, UT	1.613	Tetraploid, 2n = 32	

TABLE 1. Continued.

Species	Voucher number	Location	2C	Inferred ploidy level	Previous literature reports
<i>L. montanum</i> var. <i>chinerum</i>	8523a	Hollbrook Spring, NV	0.869	Diploid, 2n = 16	
	8523c2	Hollbrook Spring, NV	0.929	Diploid, 2n = 16	
	24136	Fredonia, AZ	3.165	Hexaploid, 2n = 64	
	8524a3	Hollbrook Ranch, NV	1.533	Tetraploid, 2n = 32	
	8524b	Hollbrook Ranch, NV	1.516	Tetraploid, 2n = 32	
<i>L. montanum</i> var. <i>coloradense</i> Rollins	8524c1	Hollbrook Ranch, NV	1.537	Tetraploid, 2n = 32	
	8517a	Gypsum, CO	1.555	Tetraploid, 2n = 32	
	8517b	Gypsum, CO	1.439	Tetraploid, 2n = 32	
	8517b	Gypsum, CO	1.439	Tetraploid, 2n = 32	
	8517c	Gypsum, CO	1.444	Tetraploid, 2n = 32	
<i>L. montanum</i> var. <i>jonesii</i> (Rydb.) C.L. Hitchc.	8537	Vernal, UT	1.486	Tetraploid, 2n = 32	Schaack et al. (1984), n = 16; Rollins and Rüdtenberg (1971), n = 16; Rollins and Rüdtenberg (1977), n = 16; Rollins (1993), 2n = 32
<i>L. montanum</i> var. <i>montanum</i>	8521c	Vernal, UT	1.219	Tetraploid, 2n = 32	
	8521c	Vernal, UT	1.479	Tetraploid, 2n = 32	
	8532	Twin Bridges along Huntington Creek, NV	1.565	Tetraploid, 2n = 32	Rollins (1993), n = 16
	8531	Whirlwind Valley, NV	1.516	Tetraploid, 2n = 32	
	8516c3	Powderhorn, CO	1.419	Tetraploid, 2n = 32	
<i>L. montanum</i> var. <i>tenellum</i> (L.O. Williams) C.L. Hitchc.	8516c3	Powderhorn, CO	1.347	Tetraploid, 2n = 32	
	71608	Mesa City, CO	1.271	Tetraploid, 2n = 32	
	71608	Mesa City, CO	2.044	Tetraploid, 2n = 32	
	71608	Mesa City, CO	1.895	Tetraploid, 2n = 32	
	71608	Mesa City, CO	2.123	Tetraploid, 2n = 32	
<i>L. montanum</i> (C.L. Hitchc.) C.L. Hitchc. var. <i>wyomingense</i>	10500	Lone Tree, WY	1.606	Tetraploid, 2n = 32	
<i>L. nanum</i> S. Wats. <i>L. osleri</i> Welsh & Goodrich <i>L. thurberii</i> Wooten	8533	Jake's Valley, NV	1.4	Tetraploid, 2n = 32	
	24080	San Francisco Mountains, UT	0.908	Diploid, 2n = 16	
	8511 dry	Lordsburg, NM	0.631	Diploid, 2n = 16	
	8510a	Columbia, NM	0.699	Diploid, 2n = 16	
	8511a	Lordsburg, NM	1.319	Tetraploid, 2n = 32	
	8511b1	Lordsburg, NM	1.316	Tetraploid, 2n = 32	
	8511c	Lordsburg, NM	1.354	Tetraploid, 2n = 32	
	10524	Table Mountain, NV	1.658	Tetraploid, 2n = 32	
	<i>L. tiehnmii</i> (Rollins) Al-Shehbaz (syn. <i>Stroganovicia tiehnmii</i> Rollins)				

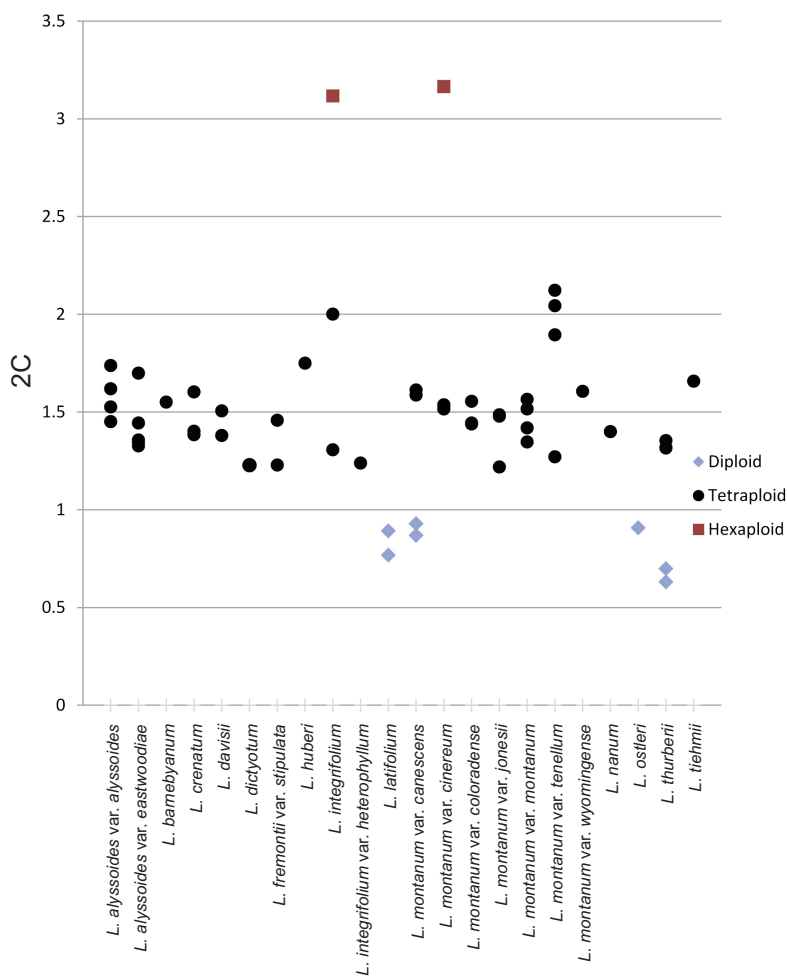


Fig. 1. Distribution of DNA content (2C) ranges across all *Lepidium* species surveyed.

variation of DNA content (2C value) of the species surveyed shows the overwhelming occurrence of tetraploids throughout the group of 14 species and 11 varieties sampled.

Occurrences of various ploidy levels were scattered across the Intermountain West. Based on the 58 samples surveyed, there are no obvious patterns of ploidy levels associated with landscape type, geographic range, or species complex (Fig. 2). Though there are differences in microhabitat preferences among most of the taxa, ploidy levels were not associated with any particular landscape or habitat feature at the collection sites of the plant material. It appears that ploidy levels, excepting tetraploids, vary randomly and are not influenced by landscape, habitat, or geographic isolation.

Though the amount of DNA may vary between species and within infraspecific taxa, there are typically no differences in ploidy level between a species and its varieties, except for *L. montanum* var. *cinereum*, which was found to have both tetraploid and hexaploid counts.

DISCUSSION

With the high abundance and widespread occurrence of polyploids, especially tetraploids, in the *Lepidium* surveyed from the Intermountain West, ploidy level does not provide much insight for explaining morphological differences or geographic patterns. However, there are a few noteworthy observations.

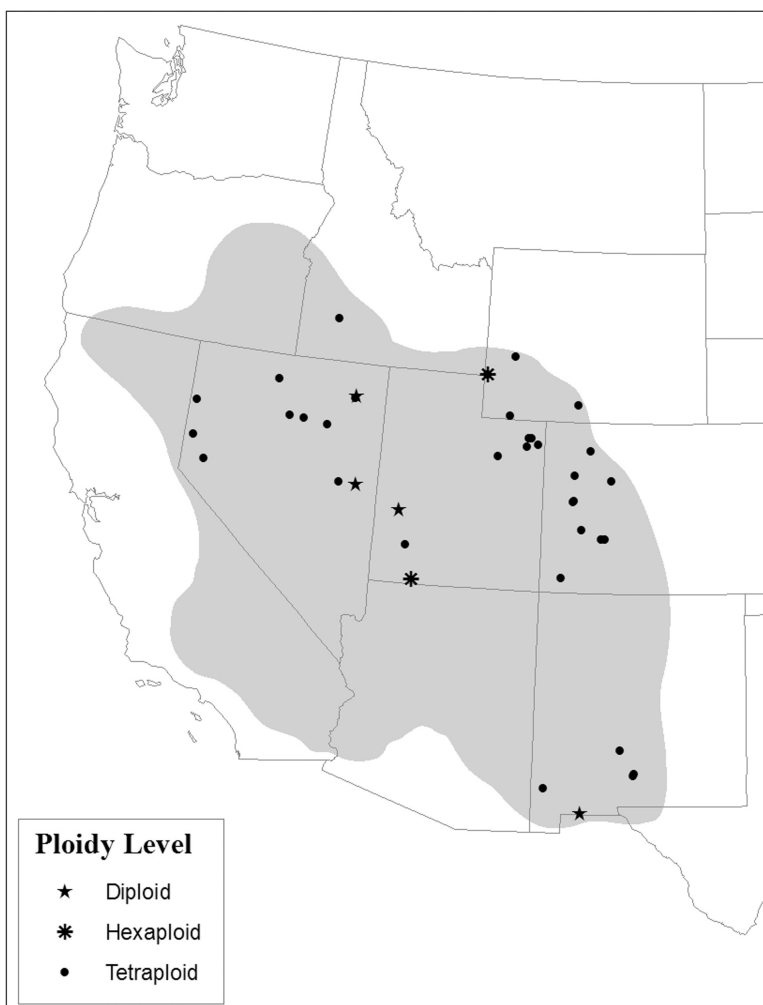


Fig. 2. Distribution of *Lepidium* with various ploidy levels in the Intermountain West.

Ploidy levels in *Lepidium* as a whole show that it is not unusual for a single taxon to have more than one ploidy level. Examples of species with more than one ploidy level are *L. latifolium* (diploid and hexaploid), *L. integrifolium* (tetraploid, hexaploid, and octoploid), *L. montanum* var. *canescens* (diploid and tetraploid), *L. montanum* var. *jonesii* (diploid [Warwick and Al-Shehbaz 2006] and tetraploid), and *L. thurberi* (diploid and tetraploid). Of the 7 varieties surveyed in the *L. montanum* complex, only var. *cinereum* and var. *canescens* had more than one ploidy level. Variety *cinereum* is reported as both tetraploid and hexaploid, and var. *canescens* as both diploid and tetraploid. The hexaploid reported for var. *cinereum*

is from a collection of var. *stellae*, which has been submerged into the concept of var. *cinereum* (Al-Shehbaz and Gaskin 2010). However, it is important to note that the results of this survey may not support a significant inference about the lack of correlation between ploidy level and taxonomic, geographic, or habitat group because the number of non-tetraploid species was so small.

Historically, the use of chromosome numbers for classification purposes in vascular plant taxonomy has long been limited to supporting or defining the concept of a species. The shape and number of chromosomes are not weighed differently than any other morphological feature for taxonomic purposes

(Stacy 1980). This is probably a result of the frequent variation in chromosome or ploidy levels. The lack of use of ploidy level to support species concepts is no doubt linked to the fact that over 70% of angiosperm plants have ploidy level increases in their evolutionary histories (Meyers and Levin 2006). Because multiple ploidy levels are common in most angiosperm groups, it would appear that little distinction among taxa could be obtained from the variability.

Stebbins (1971) proposed that a young polyploid complex would contain many diploids and a few tetraploids. As the complex ages, more species would become polyploids and higher ploidy levels would develop. As time progresses, the ancestor diploids would ultimately die out, resulting in greater difficulty in describing the evolutionary ancestry. Almost all species in this survey were of higher ploidy levels, with 90% being tetraploids. In addition, the 11 native North American species of *Lepidium* with reported chromosome counts were 90% dominated by tetraploids (Warwick and Al-Shehbaz 2006). Using Stebbins's widely accepted concept (Meyers and Levin 2006), the Intermountain West *Lepidium* complex can be described as an evolutionarily young to moderate-aged species group with a reticulate evolutionary past. The indication of a reticulate evolutionary past has been demonstrated in *Lepidium* by incongruences in gene trees (Bowman et al. 1999, Mummenhoff et al. 2001, 2004). Bowman et al. (1999) and Mummenhoff et al. (2001, 2004) suggest that most polyploids in *Lepidium* are allopolyploid. On the basis of previous cpDNA and ITS sequence data, allopolyploidization has been strongly favored to explain gene tree patterns (Mummenhoff et al. 2001, 2004). The influence of a reticulate evolutionary past in the Intermountain West region in this genus, coupled with both high occurrence of polyploidy across all species and highly mobile seeds that are easily transported by avian species to allow for mixing of populations and species (Al-Shehbaz 1986), provides for a diverse array of polyploid genotypes (Soltis and Soltis 1999). This evolutionary background may be setting the challenging stage for adequately classifying these native intermountain species.

In future research on Intermountain West *Lepidium*, DNA sequences will be analyzed and gene trees developed. Without gene trees,

these FC observations and indications of input of different parental DNA stock based on variable sizes of DNA content cannot be conclusively resolved. Likewise, with no association of geographic ploidy levels with geographic ranges or habitats and with variability of DNA content between *Lepidium* taxa, mere ploidy levels may not be useful for taxonomic purposes. However, ploidy levels do indicate the need for the construction of gene trees to adequately treat these diverse native species of *Lepidium* in the Intermountain West.

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