Basin wildrye (Leymus cinereus) and creeping wildrye (Leymus triticoides) are closely related perennial grass species (Culumber et al., 2011). Caespitose L. cinereus is considered the largest native grass in western North America, growing up to 3 m in height, and is common throughout much of western United States and Canada (Barkworth, 2007). Leymus triticoides is a shorter, strongly rhizomatous species often associated with saline meadows, but its populations are less common and somewhat more restricted to California and the Great Basin region of Nevada, Utah, and southeast Oregon (Barkworth, 2007). Both species are highly self-incompatible (Jensen et al., 1990) and known to form fertile hybrids (Dewey, 1970; Wu et al., 2003; Barkworth, 2007). Moreover, it has been suggested that the occasional presence of rhizomes in some specimens of L. cinereus may be the result of introgression from these interspecific hybrids (Barkworth, 2007). In its simplest form, Leymus Hochst. is allotetraploid (2n = 28), which is also true for both L. cinereus and L. triticoides. Octoploid (2n = 56) forms of L. cinereus and other Leymus species have also been reported (Barkworth, 2007), which may result from hybridization of divergent species (Anamthawat-Jónsson and Bödvarsdóttir, 2001) or...
autoduplication. However, the geographic distribution of different ploidy levels within *L. cinereus* has not been documented and it is not known if the octoploids are genetically different from tetraploids.

Cultivars and germplasms of *L. cinereus* are used for grazing, erosion control, and large-scale rangeland reclamation in western North America (Ogle, 2003). The cultivar Magnar (Alderson and Sharp, 1994) is believed to have originated from a natural octoploid population in southeastern British Columbia (Jones et al., 2009). The tetraploid cultivar Trailhead was originally collected near Roundup, MT, and released in 1991 (Cash et al., 1998). Continental is a cultivar (Jones et al., 2009) derived from a chromosome-doubled Trailhead population pollinated by the octoploid Magnar, which shows increased seed mass and seedling vigor compared to the parental cultivars. The *L. cinereus* germplasm Washoe (Marty 2003) was collected from a natural population growing on phytotoxic soils near the now defunct Washoe smelter stack in the Anaconda Smelter Superfund Site in western Montana, which is contaminated with As, Cd, Cu, Pb, and Zn (Marty, 2003). The *L. triticoides* cultivar Rio, released in 1991, was originally collected in Kings Valley, CA, and is used for soil stabilization in riparian areas, forage production, and reclamation of saline, irrigated croplands and pasturelands (Young-Mathews and Winslow, 2010). Both *L. cinereus* and *L. triticoides* are well adapted to alkaline and saline soils that are common throughout western North America and these plant materials are used for a variety of agricultural and conservation purposes in this region (Ogle, 2003; Barkworth, 2007; Young-Mathews and Winslow, 2010) including fire rehabilitation and other large-scale revegetation projects on federal lands, which total more than 182 million ha.

Specific laws, regulations, and executive orders support the use of native plants on USDA Bureau of Land Management and USDA Forest Service lands and there are important concerns regarding the best seed sources for these native plants (Johnson et al., 2010). Similarities in environment and climate between the site of plant-material origin and target site or sites as well as the capacity to respond and adapt to changing conditions are criterion used to determine plant material suitability (Conrad, 1983; McKay et al., 2001; Johnson et al., 2004, 2010; Jones and Monaco, 2009). Moreover, strategies have been developed to increase genetic diversity of plant materials used for reclamation by sampling plants from multiple locations representing larger genetic metapopulations, races, or “regional ecotypes” (Larson et al., 2000, 2004; Booth and Jones, 2001).

Amplified fragment length polymorphisms (AFLPs) and model-based Bayesian clustering have been used to provide an objective means of evaluating plant genetic diversity and population structure over local and broad geographical regions in western North America (Larson et al., 2004, Bhattarai et al., 2011). Neutral genetic variation detected by molecular markers provides knowledge of population structure resulting from historical effects of genetic isolation, bottlenecks, and founder effects. Some markers may also be associated with allelic variation at quantitative trait loci (QTLs) controlling adaptive trait variation (McKay and Latta, 2002). Experimental genetic mapping populations derived from interspecific hybrids of *L. cinereus* and *L. triticoides* (Wu et al., 2003) have been used to identify QTLs, genes, and DNA markers associated with adaptive traits such as plant height, caespitose and rhizomatous growth habits, and flowering traits (Larson et al., 2006), fiber, protein, and mineral content (Larson and Mayland, 2007), seed production, seed dispersal, and seed germination traits (Larson and Kellogg, 2009), and other functionally important traits such as leaf glaucousness and gamete compatibility (Larson et al., 2012). However, DNA markers used to construct these maps (Wu et al., 2003) have not been tested on diverse natural populations and very little is known about the genetic diversity and gene flow within or between these two species.

Phylogenetic analyses of chloroplast DNA sequences and multilocus nuclear AFLP genotypes demonstrated distinctively different and polyphyletic relationships involving North American and Eurasian *Leymus* species in comparisons with other Triticeae species, including wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) (Jones et al., 1999; Redinbaugh et al., 2000; Liu et al., 2008; Zhou et al., 2010; Culumber et al., 2011). In particular, it was shown that the chloroplast genome of all Eurasian *Leymus* taxa are very similar to *Psathyrostachys* Nevski (Liu et al., 2008; Zhou et al., 2010), which has been recognized as being the source of at least one of the diploid progenitors of allopolyploid *Leymus* (Dewey, 1970; Barkworth, 2007). Conversely, North American *Leymus* taxa, including *L. cinereus* and *L. triticoides*, have a unique chloroplast genome that is more similar to other Triticeae species including wheat and barley (Jones et al., 1999; Redinbaugh et al., 2000; Liu et al., 2008; Zhou et al., 2010; Culumber et al., 2011). Presumably, the chloroplast genome of North American *Leymus* comes from an unknown progenitor species within this allopolyploid genus. Therefore, previous genetic studies of *L. cinereus* and *L. triticoides* and other *Leymus* taxa have revealed an interesting phylogeographic history of perennial grasses from North America and Eurasia. However, previous studies were based on a very limited sampling of no more than six accessions from any one species (Culumber et al., 2011). The objective of this study was to sequence the same chloroplast DNA intergenic spacer sequences and multilocus AFLP markers previously used for phylogenetic studies (Culumber et al., 2011) and initial linkage mapping (Wu et al., 2003) of *Leymus* to elucidate genetic diversity and test for possible admixture within and between *L. triticoides*, *L. cinereus*, and the two ploidy levels within *L. cinereus*. 
MATERIALS AND METHODS

Plant Materials

Plant materials included 538 plants from 220 accessions of *L. cinereus*, 43 plants from 17 accessions of *L. triticoides*, and two artificial *L. cinereus × L. triticoides* F₁ hybrids, Ltc1_F1M6 and Ltc2_F1M4 (Wu et al., 2003; Larson et al., 2012) from the western United States and Canada (Supplemental Table S1). A map of these collection sites (Fig. 1) was developed using Esri ArcMap (ESRI, 2006). Included among the *L. cinereus* collections were tetraploid (2ₙ = 4ₓ = 28) cultivar Trailhead, tetraploid germplasm Washoe, and octoploid (2ₙ = 8ₓ = 56) cultivars Continental and Magnar. Samples of fresh leaf tissue harvested from one to four individual plants per accession (see Supplemental Table S1 for counts) were used (directly) for flow cytometry or desiccated in a freeze dryer and stored at −80°C before DNA extraction. The DNA was extracted from freeze-dried tissues using a MM300 mixer mill and 96-well DNAeasy system (Qiagen). Replicate DNA samples were taken from 29 *L. cinereus* plants, five *L. triticoides* plants, and one of the artificial hybrids.

Flow Cytometry

The relative DNA content of most accessions was determined using the Partec PA (I) flow cytometer (Partec). Each unknown plant was analyzed in mixture with a known tetraploid (2ₙ = 4ₓ = 28) standard, *L. cinereus* Trailhead, and in mixture with a known octoploid (2ₙ = 8ₓ = 56) standard, *L. cinereus* Magnar. Each tissue mixture was minced to release nuclei using razor blades and stained with 4’6-diamidino-2-phenylindole (DAPI) according to MATERIALS AND METHODS

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protocols of the manufacturer (Partec). Fluorescence of nuclei was measured until it was clear whether the mixture contained the same DNA content (one population peak) or different DNA content (two population peaks).

**Amplified Fragment Length Polymorphisms**

The AFLPs were carried on a total of 557 DNA samples (including replicates) as outlined in Vos et al. (1995) with the following modifications. Two selective nucleotides were used for preamplification. The selective amplification primers included five EcoRI+4/MseI+4 combinations (E.ACAc-M.CTAC, E.ACAg-M.CTTG, E.ACcA-M.CTCT, E.ACCT-M.CCTT, and E.ACgT-M.CTCT). The EcoRI-selective amplification primers included a fluorescent 6-FAM (6-carboxy fluorescein) label on 5’ nucleotides. The relative mobility of amplification products was analyzed using Applied Biosystems ABI 3730 capillary electrophoresis instruments at the Center for Integrated Biosystems (Utah State University, Logan, UT). The fragments were sized with Genescan software (Applied Biosystems, 2000) between 50 and 600 bp. The Genescan trace files were visualized and scored for the presence (1) or absence (0) of DNA bands using the software Genographer (Benham et al., 1999).

Genetic relationships among individual plants and accessions were tested using both model-based and distance–based clustering methods. Individual plants were clustered into genetic populations using the model-based Bayesian clustering analysis, allowing for dominant allele admixture, without an assignment of flags for hierarchical groups using the STRUCTURE v2.3 program (Falush et al., 2003). The statistical significance of genetic variability within and between groups was also compared using the nonparametric AMOVA permutation test (Excoffier et al., 2005) based on two-parameter estimates of the average number of nucleotide differences per site between individual plants (Kimura, 1980). Phylogenetic analyses of intergenic spacer haplotypes were performed with heuristic parsimony searches with simple sequence addition in PAUP* version 4.0b10 (Swofford, 2002). Bootstrap-support values for parsimony analyses were obtained from 1000 heuristic searches with simple addition and no time limit and 50% consensus threshold. Sequences from Eurasian ‘Mustang’ A. wildrye [Leymus angustus (Trin.) Pilg.] and ‘Volga’ mammoth wildrye [Leymus racemosus (Lam.) Tzvelev] were included as outgroups for chloroplast DNA analysis (Culumber et al., 2011).

**Chloroplast DNA**

Noncoding chloroplast DNA regions were amplified and sequenced using the trnH-psbA and trnK-rps16 primers. Approximately 30 ng of plant DNA was amplified in 25-μL volumes (10 mM Tris-HCl [pH 9.0], 50 mM KCl, 0.1% Triton X-100, 2.5 mM MgCl₂, 2.0 mM deoxyribonucleotide triphosphates, 10 μM primers, and 1 U Taq DNA polymerase). The two-step temperature profile for polymerase chain reaction (PCR) included 94°C for 1 min; 5 cycles of 94°C denaturing for 30 s, annealing at 53°C for 45 s, and 72°C extension for 1 min 30 s followed by 30 cycles 94°C denaturing for 30 s, annealing at 48°C for 45 s, and 72°C for 1 min; and finally a 72°C extension for 7 min. The Quickstep 2 PCR and the ExcelaPure 96-well UF PCR purification kits (Edge-Biosystems) were used to purify PCR products before sequencing. Chloroplast PCR product size ranged from 600 to 650 bp, requiring 0.6 μL of purified PCR product, and 1 μL of 2 pmol μL⁻¹ primer (2 μM) for each 10 μL sequencing reaction. Sequencing reactions were performed according to Applied Biosystems Big Dye terminator v3.1 cycle–sequencing protocol. Finally, sequencing products were purified with PerformA V3 96-well Short Plates (Edge-Biosystems). The retained elutes from this final purification were loaded onto the ABI3730 for sequence analysis. Complementary strands for each sample were aligned and manually inspected in SEQUENCER 4.5 and 4.6 (Gene Codes, 2005, 2006). Concatenated consensus sequences from the trnH-psbA and trnK-rps16 amplicons (Soltis et al., 1996; McKenzie et al., 2006) were aligned in MEGA4 (Tamura et al., 2007).

The statistical significance of chloroplast DNA variability within and between groups was also compared using the nonparametric AMOVA permutation test (Excoffier et al., 2005) based on two-parameter estimates of the average number of nucleotide differences per site between individual plants (Kimura, 1980). Phylogenetic analyses of intergenic spacer haplotypes were performed with heuristic parsimony searches with simple sequence addition in PAUP* version 4.0b10 (Swofford, 2002). Bootstrap-support values for parsimony analyses were obtained from 1000 heuristic searches with simple addition and no time limit and 50% consensus threshold. Sequences from Eurasian ‘Mustang’ A. wildrye [Leymus angustus (Trin.) Pilg.] and ‘Volga’ mammoth wildrye [Leymus racemosus (Lam.) Tzvelev] were included as outgroups for chloroplast DNA analysis (Culumber et al., 2011).

**RESULTS**

**Flow Cytometry**

Stained-nuclei preparations of all L. triticoides accessions and 87 of the L. cinereus accessions were deemed to be tetraploid and 128 of the L. cinereus accessions were deemed to be octoploid (Table 1; see also Supplemental Table S1 for each accession). The octoploid accessions were more abundant in the western range of the L. cinereus sample distribution whereas the tetraploid accessions were more abundant in the east (Fig. 1). Both octoploid and tetraploid accessions were common in the southwestern range of the L. cinereus sample distribution (Fig. 1); however, mixed ploidy levels were not found within accessions.
Amplified Fragment Length Polymorphism Variation within and between Species and Accessions

A total of 807 bands were detected over all seven primer combinations that ranged in size from about 66 to 480 bp in length. The average number of bands per plant over all seven primer combinations was 100.0 (SE = 0.06) or about 14 bands per plant per primer. However, the average number of bands per plant in octoploid *L. cinereus* accessions, 110.3 (SE = 0.5), was significantly higher than tetraploid *L. cinereus* accessions, 87.4 (SE = 0.5), or tetraploid *L. triticoides* accessions (Table 1). A total of 14 *L. triticoides* bands were monomorphic (present in more than 95% of samples, excluding known hybrids), 12 *L. cinereus* bands were monomorphic, and two bands were monomorphic in both taxa. Seven of the 14 monomorphic *L. triticoides* bands were also present in more than 5% of the *L. cinereus* samples. Conversely, 10 of the 12 monomorphic *L. cinereus* bands were also present in more than 5% of the *L. triticoides* samples. A total of 283 *L. cinereus* bands and 221 *L. triticoides* bands were polymorphic (present and absent in more than 5% of samples). As expected, the average Dice similarity coefficient (proportion of shared bands) among replicated DNA samples, 0.973 (SE = 0.005), was much higher than the average similarity coefficient within accessions, 0.673 (SE = 0.004). The maximum similarity coefficient between different plants was 0.825, indicating that no two plants were genetically identical. As expected, the average Dice similarity coefficients between species, 0.169, was significantly lower than the average similarity coefficients within species (Table 1).

The average number of differences among *L. cinereus* accessions (92.8) was about 33.1% greater (*P* ≤ 0.001) than the average number of differences within *L. cinereus* accessions (62.1). The average number of differences among *L. triticoides* accessions (73.0) was about 51.1% greater (*P* ≤ 0.001) than the average number of differences within *L. triticoides* accessions (35.7). The average total number of differences between *L. cinereus* and *L. triticoides* (152.1) was about 42.9% greater (*P* ≤ 0.001) than the average number differences within species. Compared to *L. triticoides*, *L. cinereus* maintains relatively high DNA variation within accessions (66.9%) and is more genetically diverse overall. Moreover, linkage disequilibrium was more prevalent in *L. cinereus*, suggesting it has more population structure than *L. triticoides* (Table 1).

Genetic and geographic distances among 217 *L. cinereus* accessions were significantly correlated, but the correlation coefficient was low (Table 1). A similar trend was observed among 20 *L. triticoides* accessions (Table 1), but it was not statistically significant.

Bayesian Analysis of Amplified Fragment Length Polymorphism Genotypes

Bayesian clustering analysis detected genetic admixture and genetic structure within and between species. The Bayesian model probabilities showed a sharp increase from *K* = 1 to *K* = 2 groups (Fig. 2), which was expected given the high levels of genetic differentiation between *L. triticoides* and *L. cinereus*. The *K* = 2 Bayesian cluster analysis primarily separates *L. triticoides* and *L. cinereus* accessions, as do all other multiple-group model tests. However, the two-group Bayesian model test and all other model tests suggested low levels of genetic admixture between species. The 43 plants classified as *L. triticoides* had *L. cinereus* ancestry coefficients ranging from 0.001 to 0.073 (Fig. 3), with an overall average of 0.007 (0.7% admixture), in the range of genetic admixture between species. Conversely, 536 plants classified as *L. cinereus* had *L. triticoides* ancestry coefficients ranging from 0.000 to 0.759 (Fig. 3), with an overall average of 0.009

Table 1. Comparisons of number of octoploid and tetraploid accessions, average numbers of amplified fragment length polymorphism (AFLP) bands, Dice similarity coefficients based on AFLP profiles, linkage disequilibrium among AFLP markers, and correlations between genetic and geographical distance.

<table>
<thead>
<tr>
<th></th>
<th>Overall</th>
<th>Rocky Mountain race</th>
<th>Columbia race</th>
<th>Great Basin race</th>
<th>Leymus triticoides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number accessions</td>
<td>217</td>
<td>88</td>
<td>107</td>
<td>22</td>
<td>17</td>
</tr>
<tr>
<td>4x tetraploids</td>
<td>87</td>
<td>72</td>
<td>10</td>
<td>5</td>
<td>17</td>
</tr>
<tr>
<td>8x octoploids</td>
<td>128</td>
<td>15</td>
<td>97</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Number AFLP bands (SE)</td>
<td>102.2 (0.6)</td>
<td>89.4 (0.6)</td>
<td>111.7 (0.5)</td>
<td>98 (1.0)</td>
<td>80.3 (0.9)</td>
</tr>
<tr>
<td>Dice similarity coefficient</td>
<td>0.547**</td>
<td>0.556**</td>
<td>0.601**</td>
<td>0.619**</td>
<td>0.566**</td>
</tr>
<tr>
<td>Percentage LD†</td>
<td>21.5</td>
<td>8.3</td>
<td>8.5</td>
<td>5.0</td>
<td>11.1</td>
</tr>
<tr>
<td>Correlation genetic vs.</td>
<td>0.297**</td>
<td>NS†</td>
<td>0.187***</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>geographic distances</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**Significant at the 0.01 probability level.
***Significant at the 0.001 probability level.
†LD, linkage disequilibrium; percentage of comparisons among 79 common markers showing LD.
§NS, nonsignificant.
A total of 49 (9.1%) of the 536 *L. cinereus* plants had *L. triticoides* ancestry coefficients greater than 0.010 (more than 1% admixture), respectively (Fig. 3). A total 13 (2.4%) of the 536 plants classified as *L. cinereus* and two artificial F1 hybrids showed more than 10% admixture between species (Fig. 3). Only 60 (11.1%) of the 536 *L. cinereus* plants showed pure species ancestry (Fig. 3). Plants of the *L. cinereus* accession ‘NV62’ (Supplemental Table S1) showed *L. triticoides* ancestry coefficients of 0.503 and 0.759. The two artificial *L. triticoides × L. cinereus* hybrids,
Ltc1_F1M06 and Ltc2_F1M04, had *L. triticoides* ancestry coefficients of 0.504 and 0.559, respectively. Although it is difficult to distinguish true admixture (resulting from hybridization between species) from shared ancestral polymorphisms (resulting from common ancestor), these putative introgressions were not randomly distributed across the landscape (Fig. 1). All but one of the *L. cinereus* accessions showing greater than 1% admixture were collected from regions of northwestern California, southeastern Oregon, and southwestern Idaho where *L. triticoides* is common (Fig. 1; see Supplemental Table S1 for values). Conversely, *L. cinereus* plants north of the 45th parallel (equivalent to the border between Wyoming and Montana) (Fig. 1), showed less than 0.18% admixture (maximum of 0.19%), which was significantly lower than average (P ≤ 0.01). Of the 17 *L. cinereus* accessions showing greater than 1% admixture, eight were tetraploid and nine were octoploid (Fig. 1; see Supplemental Table S1 for values). Wildland seed of the *L. cinereus* Lcin_NV.24 accession, with *L. triticoides* ancestry coefficient of 0.239, was collected in the immediate vicinity of *L. triticoides*.

Bayesian cluster analysis detected genetic structure within *L. cinereus*; however, all *L. triticoides* accessions held together in one group from K = 2 to K = 10. Bayesian model probabilities also showed a relatively large increase from K = 2 to K = 3 and from K = 3 to K = 4, but probabilities reached a plateau and become more variable from K = 4 to K = 10. The ancestry coefficients for each accession from the K = 4 model, including three *L. cinereus* groups and one monotypic *L. triticoides* group, were graphically displayed by geographical locality (Fig. 1). The three *L. cinereus* Bayesian gene pools have different geographic distributions (Fig. 1), which generally correspond to the Columbia, Rocky Mountain, and Great Basin regions. The correlations of genetic and geographical distances within these three *L. cinereus* Bayesian groups were weak compared to correlations over all *L. cinereus* accessions (Table 1). Therefore, these three *L. cinereus* groups evidently account for most of the geogenetic structure in *L. cinereus* and were named after the Columbia, Rocky Mountain, and Great Basin regions to which they correspond (Fig. 1). The Columbia race extends from British Columbia in the north, south through the Columbia River Plateau of Washington and Oregon, and farther south into the Sierra Steppe of southeastern Oregon and northern California (Fig. 1). The Rocky Mountain race extends from the Rocky Mountain Piedmont of Alberta and Montana in the north, south through Wyoming, Utah, and Colorado, and west across the Snake River Plateau of Idaho and the Intermountain region of Nevada and Utah (Fig. 1). The Great Basin race is interspersed with the Rocky Mountain accessions, but it is restricted to the Great Basin region of southwestern Idaho, Nevada, and western Utah (Fig. 1).

Hierarchical AMOVA within *L. cinereus* apportioned about 14.4% of the DNA polymorphism among the three *L. cinereus* races (P ≤ 0.001) and about 26.8% among accesses within races (P ≤ 0.001). A principal component analysis (PCA) plot based on the average genetic distances among accessions was developed to show genetic variation within and among the three geographical races of *L. cinereus* (Fig. 4). The Great Basin race is most similar to the Rocky Mountain race on the first PCA axis (Fig. 4) and shows greater overall divergence from the Columbia race (Table 2). The apportionment of total variation between species was much greater than the apportionment of variation among *L. cinereus* races (Table 2). Hierarchical AMOVA of both *L. cinereus* and *L. triticoides* apportioned 38.9% of the DNA polymorphism among species and 7.1% among races. Linkage disequilibrium among AFLP markers was considerably less frequent within races compared to disequilibrium over all *L. cinereus* accessions, suggesting that these races reflect a major portion of the genetic structure within this species.

Genetic differences and admixture between *L. cinereus* and *L. triticoides* were not evenly distributed among the three geographic races of *L. cinereus*. Compared to the other two races, the Great Basin race showed greater divergence from *L. triticoides* (Table 2). Conversely, genetic admixture between *L. triticoides* and the Great Basin race (average 0.24%) was less than corresponding values in the Rocky Mountain race (average 0.45%) or Columbia race (average 1.03%). One accession, Lcin_NV.24, which was predominantly Rocky Mountain race (55.8%), also showed relatively high admixture with *L. triticoides* (23.3%) and the Great Basin race (20.5%). However, admixture between *L. triticoides* and other accessions belonging to the Rocky Mountain race was generally low (average 0.19%). Admixture between *L. cinereus* and *L. triticoides* was significantly higher (P ≤ 0.001) among the 26 Columbia race accessions south of the 45th parallel (3.67%) than the other 75 (northern) Columbia race accessions (0.18%) or the other two races.

**Amplified Fragment Length Polymorphism Genotypes of Basin Wildrye and Creeping Wildrye Cultivars**

The ancestry coefficients of tetraploid Trailhead and Washoe *L. cinereus* cultivars were 95.9 and 94.8% Rocky Mountain race, respectively, based on the K = 4 Bayesian cluster analysis. The ancestry coefficient of octoploid Magnar *L. cinereus* cultivar was 98.0% Columbia race. The ancestry coefficient of the octoploid Continental *L. cinereus* cultivar, derived from hybrids of Trailhead × Magnar, correspond to 56.2% Rocky Mountain race and 43.4% Columbia race. The mixed ancestry of Continental was also evident in the PCA plot, which placed it midway between its parents Trailhead and Magnar (Fig. 4). Rio *L. triticoides* contained less than 0.2% *L. cinereus* ancestry.

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**Table 1.** Summary of admixture analysis results. The ancestry coefficients for each accession from the K = 4 model, including three *L. cinereus* groups and one *L. triticoides* group, were graphically displayed by geographical locality (Fig. 1). The three *L. cinereus* Bayesian gene pools have different geographic distributions (Fig. 1), which generally correspond to the Columbia, Rocky Mountain, and Great Basin regions. The correlations of genetic and geographical distances within these three *L. cinereus* Bayesian groups were weak compared to correlations over all *L. cinereus* accessions (Table 1). Therefore, these three *L. cinereus* groups evidently account for most of the geogenetic structure in *L. cinereus* and were named after the Columbia, Rocky Mountain, and Great Basin regions to which they correspond (Fig. 1). The Columbia race extends from British Columbia in the north, south through the Columbia River Plateau of Washington and Oregon, and farther south into the Sierra Steppe of southeastern Oregon and northern California (Fig. 1). The Rocky Mountain race extends from the Rocky Mountain Piedmont of Alberta and Montana in the north, south through Wyoming, Utah, and Colorado, and west across the Snake River Plateau of Idaho and the Intermountain region of Nevada and Utah (Fig. 1). The Great Basin race is interspersed with the Rocky Mountain accessions, but it is restricted to the Great Basin region of southwestern Idaho, Nevada, and western Utah (Fig. 1).

**Table 2.** Hierarchical AMOVA analysis results. The apportionment of total variation between species was much greater than the apportionment of variation among *L. cinereus* races (Table 2). Hierarchical AMOVA of both *L. cinereus* and *L. triticoides* apportioned 38.9% of the DNA polymorphism among species and 7.1% among races. Linkage disequilibrium among AFLP markers was considerably less frequent within races compared to disequilibrium over all *L. cinereus* accessions, suggesting that these races reflect a major portion of the genetic structure within this species. Genetic differences and admixture between *L. cinereus* and *L. triticoides* were not evenly distributed among the three geographic races of *L. cinereus*. Compared to the other two races, the Great Basin race showed greater divergence from *L. triticoides* (Table 2). Conversely, genetic admixture between *L. triticoides* and the Great Basin race (average 0.24%) was less than corresponding values in the Rocky Mountain race (average 0.45%) or Columbia race (average 1.03%). One accession, Lcin_NV.24, which was predominantly Rocky Mountain race (55.8%), also showed relatively high admixture with *L. triticoides* (23.3%) and the Great Basin race (20.5%). However, admixture between *L. triticoides* and other accessions belonging to the Rocky Mountain race was generally low (average 0.19%). Admixture between *L. cinereus* and *L. triticoides* was significantly higher (P ≤ 0.001) among the 26 Columbia race accessions south of the 45th parallel (3.67%) than the other 75 (northern) Columbia race accessions (0.18%) or the other two races.
Identification of Amplified Fragment Length Polymorphism Markers for Discrimination of Species and Races

Of the 807 AFLP markers scored, a total of 52 private alleles were detected in *L. triticoides* and 418 private alleles detected in *L. cinereus*, excluding a relatively small number of samples that were less than 90% pure based on the Bayesian cluster analysis (Fig. 3). However, none of these private alleles were fixed within the host species. Likewise, a total of 88, 81, and 27 private alleles were detected in the Columbia, Rocky Mountain, and Great Basin races, respectively, after exclusion of about 10% of the samples that showed more than 10% admixture among races. However, most of these private alleles were rare with maximum frequencies of 0.20, 0.18, and 0.29 within these races. One marker was present among 85% of the *L. cinereus* samples, but the next best *L. cinereus* marker was only present in 64% of the individuals sampled from this species. Two markers were present among nearly 94 and 77% of the *L. triticoides* samples, but the third best *L. triticoides* marker was present in only 58% of the *L. triticoides* samples.

The most discriminatory markers present were relatively abundant within groups and rare or occasionally absent in other groups. We identified a panel of 30 discriminatory markers that can effectively classify 97% of the *L. cinereus* plants into the same group (race), by unweighted pair group method with arithmetic mean (UPGMA) cluster analysis, as was determined by the Bayesian cluster analysis using the complete set of markers (Supplemental Table S2). Likewise, we identified a list of 20 markers that were abundant within each species and rare in the other. However, only four or five of these markers were really necessary to correctly classify individuals by species by UPGMA analysis.

Chloroplast DNA Haplotypes

The chloroplast *trnH-psbA* and *trnK-psi6* intergenic DNA sequences from 199 *L. cinereus* and 14 *L. triticoides* plants collapsed into 22 haplotypes (see Supplemental Table S1).
for haplotype and GenBank identifiers). Eighteen occurred only in *L. cinereus*, two haplotypes were common to both *L. cinereus* and *L. triticoides* (Table 3). The total aligned sequence length was 1331 bp across the 22 haplotypes and two outgroup taxa, *L. racemosus* and *L. angustus*. However, four regions of the alignment comprising 115 bp (381–448, 794–800, 825–845, and 1223–1241) were eliminated from further analysis due to ambiguous indels and a 6 bp palindromic repeat. The remaining alignment included a total of 1177 constant characters, 30 variable nucleotide sites, and nine variable gap code characters. The four most common chloroplast DNA haplotypes (CPH40, CPH41, CPH42, and CPH44) made up 181 (85%) of the 213 plants (Table 3), including all released *L. cinereus* plant materials. The *L. cinereus* and *L. triticoides* haplotypes differed by one to nine steps, but the four most common haplotypes differed by only one to four steps. A heuristic parsimony tree was obtained for these 22 *L. cinereus* and *L. triticoides* haplotypes, rooted using *L. racemosus* and *L. angustus* outgroups, but it does not effectively distinguish these species or races (Supplemental Fig. S1).

Unexpectedly, chloroplast DNA divergence between the Great Basin and Columbia races, measured by total differences and the apportionment of variation between groups, was higher than divergence between the Great Basin race and *L. triticoides* (lower diagonal in Table 4). One trend that is similar between AMOVAs of AFLP markers (Table 2) and chloroplast DNA sequences (Table 4) is that the Great Basin and Columbia races show greater divergence than the Great Basin and Rocky Mountain races. Hierarchical AMOVA of the chloroplast DNA variation detected 8.0% divergence between species and 11.0% among races.

**DISCUSSION**

Bayesian clustering analysis of AFLP genotypes detected at least three divergent races within *L. cinereus*, providing new evidence that this is a polytypic species whereas *L. triticoides* was consistently monotypic in this analysis. Released plant materials Trailhead, Washoe, Magnar, and Continental are included among two of the three natural *L. cinereus* races. The majority of accessions of the Rocky Mountain race, including Trailhead and Washoe, are tetraploid. The majority of accessions of the Columbia race, including cultivar Magnar, are octoploid. According to this model, Continental is hybrid of the Rocky Mountain and Columbia races, which is consistent with the pedigree of this germplasm. Plant materials representing a third race, from the Great Basin region, have not been released.

One of the key assumptions in the model-based Bayesian clustering is that sampling of individuals is random with regard to the population as a whole, regardless of where individuals might be located (Pritchard et al., Table 3. Frequency of chloroplast trnH-psbA and trnK-rps16 intergenic DNA haplotypes tabulated by species and race. The percentages in parentheses indicate frequency among all accessions.

<table>
<thead>
<tr>
<th>Chloroplast DNA haplotype (CPH)</th>
<th>Overall</th>
<th>Rocky Mountain race</th>
<th>Columbia race</th>
<th>Great Basin race</th>
<th>Leymus triticoides</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPH09 1 (0.5%)</td>
<td>0 (0%)</td>
<td>1 (1%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>CPH11 1 (0.5%)</td>
<td>1 (1.3%)</td>
<td>0 (0%)</td>
<td>1 (5.3%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
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<tr>
<td>CPH12 1 (0.5%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>1 (5.3%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>CPH13 1 (0.5%)</td>
<td>1 (1.3%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>CPH14 1 (0.5%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>1 (5.3%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>CPH15 1 (0.5%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>1 (5.3%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
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<tr>
<td>CPH18 1 (0.5%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>1 (5.3%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
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<tr>
<td>CPH28 1 (0.5%)</td>
<td>0 (0%)</td>
<td>1 (1%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>CPH30 7 (3.5%)</td>
<td>4 (5%)</td>
<td>0 (0%)</td>
<td>3 (15.8%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
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<tr>
<td>CPH36 3 (1.5%)</td>
<td>3 (3.8%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
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<tr>
<td>CPH37 1 (0.5%)</td>
<td>1 (1.3%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>CPH39 1 (0.5%)</td>
<td>1 (1.3%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>CPH40 42 (21.1%)</td>
<td>29 (36.3%)</td>
<td>12 (12%)</td>
<td>1 (5.3%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
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<tr>
<td>CPH41 84 (42.2%)</td>
<td>35 (43.8%)</td>
<td>42 (42%)</td>
<td>7 (36.8%)</td>
<td>4 (28.6%)</td>
<td>0 (0%)</td>
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<tr>
<td>CPH42 27 (13.6%)</td>
<td>0 (0%)</td>
<td>27 (27%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
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<tr>
<td>CPH44 16 (8%)</td>
<td>5 (6.3%)</td>
<td>8 (8%)</td>
<td>3 (15.8%)</td>
<td>8 (57.1%)</td>
<td>0 (0%)</td>
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<tr>
<td>CPH46 0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>1 (7.1%)</td>
</tr>
<tr>
<td>CPH47 0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>1 (7.1%)</td>
</tr>
<tr>
<td>CPH48 5 (2.5%)</td>
<td>0 (0%)</td>
<td>5 (5%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>CPH49 3 (1.5%)</td>
<td>0 (0%)</td>
<td>2 (2%)</td>
<td>1 (5.3%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>CPH56 1 (0.5%)</td>
<td>0 (0%)</td>
<td>1 (1%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Totals 199 (99.9%)</td>
<td>80 (100.4%)</td>
<td>100 (100%)</td>
<td>19 (100.2%)</td>
<td>14 (100%)</td>
<td></td>
</tr>
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</table>
Table 4. The apportionment of chloroplast trnH-psbA and trnK-rps16 intergenic DNA sequence variation, measured by Kimura’s two-parameter nucleotide substitution rate (and the number of mutation steps), among three Leymus cinereus races and Leymus triticoides. Above diagonal: the average total sequence variation between individual plants of different groups. Diagonal (bold): the average sequence variation between individual plants within groups. Below diagonal: the apportionment of sequence variation between groups tested by analysis of molecular variance. The percentages indicate the proportion of variation relative to all race comparisons.

<table>
<thead>
<tr>
<th></th>
<th>Rocky Mountain L. cinereus</th>
<th>Columbia L. cinereus</th>
<th>Great Basin L. cinereus</th>
<th>L. triticoides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rocky Mountain</td>
<td>7.7 (1.49)</td>
<td>11.3 (1.89)</td>
<td>14.4 (2.24)</td>
<td>8.9 (1.51)</td>
</tr>
<tr>
<td>Columbia</td>
<td>15.3%***</td>
<td>11.3 (1.89)</td>
<td>18.6 (2.72)</td>
<td>12.1 (1.89)</td>
</tr>
<tr>
<td>Great Basin</td>
<td>5.2%**</td>
<td>18.0%***</td>
<td>20.5 (2.73)</td>
<td>15.5 (2.24)</td>
</tr>
<tr>
<td>L. triticoides</td>
<td>18.3%**</td>
<td>21.9%***</td>
<td>10.8%*</td>
<td>6.7 (2.00)</td>
</tr>
</tbody>
</table>

*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level.
***Significant at the 0.001 probability level.

2000). If sampling is not random, geographic gaps in the sampling of individuals could give rise to genetic clusters caused by discontinuous sampling, even if allele frequencies vary continuously across the landscape (Pritchard et al., 2000). Although many L. cinereus sites were sampled within the central range of this species (Fig. 1), this area includes large and remote desert and mountain habitats that were not sampled. In particular, a large gap in our sampling of L. cinereus is evident in the northern Rocky Mountain region (Fig. 1) even though we know that L. cinereus is present throughout this mountainous region. Nevertheless, all three genetically distinct L. cinereus races were detectable near the intersection of Oregon, Idaho, and Nevada. Although correlations between genetic and geographic distances over all L. cinereus accessions were largely attributed to differences among these three geographic races, we do not believe that these genetic differences were an artifact of discontinuous sampling or any currently existing barriers among these three races. However, it is possible and likely that these three races may have been geographically isolated during Pleistocene glacial cycles. The sparse sampling of L. triticoides (Fig. 1) reflects a number of factors including natural dispersion of L. triticoides populations (Barkworth, 2007), the inconspicuous nature of L. triticoides, and the poor seed production of natural L. triticoides swards. Nevertheless, gaps between L. triticoides sampling sites did not create artificial genetic groups.

Conversely, incomplete sampling from the peripheral range of species distribution could result in failure to detect biologically significant groups (Cassel-Lundhagen et al., 2009; Wagner et al., 2011). Once a dominant species of the Central Valley before European settlement, L. triticoides is still found throughout the entire state of California, northern Arizona, and the eastern Rocky Mountain front ranges of New Mexico, Colorado, Wyoming, and Montana (Barkworth, 2007; Young-Mathews and Winslow, 2010). More extensive sampling of L. triticoides might reveal groups that were not detected in this study (Fig. 1). Similarly, the natural distribution of L. cinereus extends throughout most of Wyoming and the mountainous country of western Colorado, northern Arizona, and the entire Sierra Nevada mountain range of California (Barkworth, 2007), which may contain other L. triticoides races not detected here. Although the Great Basin race of L. cinereus appears to be sympatric within the more widely distributed Rocky Mountain race (Fig. 1), it is entirely possible that the so-called Great Basin race extends beyond the sampling range of this study.

Relatively few plants showed pure-species ancestry without any possible admixture or introgression between L. cinereus and L. triticoides, but only 13 (2.2%) of the 579 natural samples showed greater than 10% putative admixture between species. One accession classified as L. cinereus (Rocky Mountain race) showed L. triticoides ancestry coefficients of 0.504 and 0.759 (Fig. 3), but this can be explained by the observation that some conspicuous L. cinereus traits, such as tall plant height and caespitose growth, are dominant or at least partially dominant (Larson et al., 2006). Aside from the 13 plants showing relatively high levels of admixture, it may be difficult to distinguish true genetic admixture resulting from hybridization and ancestral shared polymorphisms resulting from population splits with incomplete lineage sorting (Sousa et al., 2012). However, this putative genetic admixture between L. cinereus and L. triticoides was not randomly distributed across the landscape. In fact, most of the high-level and low-level admixture between L. cinereus and L. triticoides was concentrated in the southern portion of the L. cinereus Columbia race, south of the 45th parallel, where these species are sympatric, and it included a mix of tetraploid and octoploid accessions. Aside from a few other early-generation hybrids, admixture in other races and regions was very low (less than 0.24%). Admixture was very low north of the 45th parallel where the distribution of L. triticoides is very sparse (Barkworth, 2007). The nonrandom geographic distribution of genetic admixture, detected by AFLP markers, suggests that the admixture occurred after divergence of these species and races rather than shared polymorphisms inherited from a common ancestor. More extensive genotyping of these putative admixed populations is needed to determine if the introgressed marker alleles are associated with adaptively important QTLs markers, as suggested by Lexer et al. (2004), identified using experimental mapping populations derived from L. triticoides × L. cinereus hybrids (Larson et al., 2006, 2012; Larson and Mayland, 2007; Larson and
Kellogg, 2009). Moreover, both L. cinereus and L. triticoides may hybridize with other species not included in this study. Giant wildrye [Leymus condensatus (J. Presl) Å. Löve] is distributed throughout the coastal ranges of western California and also forms natural hybrids with L. triticoides, known as Leymus multimicrops (Gould) Barkworth & R. J. Atkins (many-flowered wildrye) (Stebbins and Walters, 1948; Barkworth, 2007). Similarly, Leymus ambiguus (Vasey & Scribn.) D. R. Dewey (Colorado wildrye) is thought to be a hybrid of L. cinereus and Leymus salina (M. E. Jones) Å. Löve (Salina wildrye) (Culumber et al., 2011). Therefore, there is ample evidence that hybridization among North American Leymus taxa may be an important evolutionary factor.

The chloroplast psbA-trnH and rps16-trnK sequences provided clear discrimination between Eurasian and North American groups of Leymus (Culumber et al., 2011). Although significant differences in the average number of chloroplast DNA differences can be detected among a priori defined groups, these chloroplast DNA markers are not of practical use for distinguishing L. triticoides and the three races of L. cinereus. We hypothesize that the lack of chloroplast DNA divergence among North American Leymus taxa is the result of recent divergence and shared ancestral polymorphisms.

Although AFLPs provided clear discrimination of species and effective identification of three races within L. cinereus, these differences were not the result of discrete differences among marker genotypes of these groups. Most of the private alleles within species and races were also relatively rare within these host groups. We found that at least 30 of the 755 markers detected in L. cinereus were required to correctly classify individual plant by race. Although it appears to be possible to identify species using a relatively small number of markers, this was not the case for identification of races within L. cinereus. These patterns of AFLP differentiation are also consistent with recent divergence between species and incomplete divergence among races.

Supplemental Information Available
Supplemental material is included with this manuscript.

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References


