

DNA BARCODING OF WESTERN NORTH AMERICAN TAXA:

LEYMUS (POACEAE) AND *LEPIDIUM* (BRASSICACEAE)

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Ecology

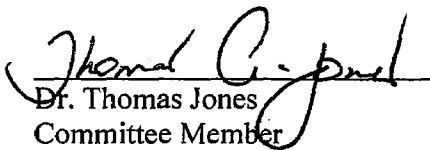
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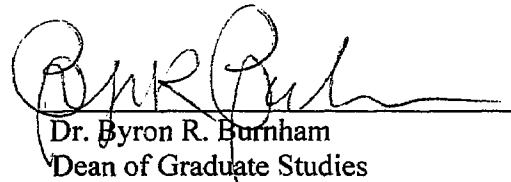
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ABSTRACT

DNA Barcoding of Western North American Taxa:

Leymus (Poaceae) and *Lepidium* (Brassicaceae)

by

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My objective was to determine if polymorphic information from the 18S-5.8S-26S nuclear ribosomal DNA internal transcribed spacer regions and the *trnK-psbA*, *trnK-rps16* chloroplast DNA spacer regions is sufficient 1) to identify a plant specimen to the species level, and 2) to establish the phylogenetic relationship between species. The first study examined the relationship of various North American as well as European and Asian species of perennial, *Leymus* wildrye grasses (Poaceae). Three North American *Leymus* taxa, including *L. flavescens*, *L. innovatus*, and *L. mollis*, displayed unique haplotypes in both chloroplast DNA and internal transcribed spacer sequences. However, this specific set of DNA barcodes was insufficient to unambiguously identify individual plants in *L. cinereus* and *L. triticoides*, the foremost taxa in the sample set. Chloroplast DNA phylogenies separated North American and Eurasian *Leymus* species into two distinct groups, with an estimated divergence time of $.65 \times 10^6$ to 2.3×10^6 million years ago. The Eurasian and North American *Leymus* cpDNA sequences are most like

Psathyrostachys and *Thinopyrum* reference taxa, respectively, which have been suggested as probable diploid ancestors of polyploidy *Leymus*.

The second study analyzed the relationship between two Brassicaceae species. The proposed endangered species *Lepidium papilliferum* was compared to *Lepidium montanum*, a species with proximal distribution, similar morphology, life history traits, and habitat. One mutation distinguished all *L. papilliferum* from all but three *L. montanum* accessions. Significant levels of genetic differentiation were found for chloroplast ($F_{st} = 0.11660$), and the internal transcribed spacer ($F_{st} = 0.33778$) between *L. montanum* and *L. papilliferum* based on the Kimura's 2-parameter test of sequence divergence. Divergence time estimates between *L. montanum* and *L. papilliferum* range from 22 400 to 10 400 years ago, based on a 0.008% nucleotide-sequence divergence between species for the chloroplast DNA sequences, and 136 000 to 74 000 years ago based on a 0.124% nucleotide sequence divergence for internal transcribed spacer sequences. The recent divergence times suggest a very close relationship between *L. papilliferum* and *L. montanum*. Additional sampling with less geographical bias may reveal more continuous relationships between *L. montanum* and *L. papilliferum*.

ACKNOWLEDGMENTS

Funding for this thesis research was provided by the Great Basin Native Plant Selection and Increase project, a multi-state, research project formed in collaboration of the U.S. Department of the Interior-Bureau of Land Management Great Basin Restoration Initiative, the U.S. Department of Agriculture Forest Service Rocky Mountain Research Station Shrubland Biology and Restoration Research Work Unit, and other collaborators, including the Utah Division of Wildlife Resources – Pittman / Robertson Big Game Habitat Restoration Project W-82-R, and the U.S Department of Agriculture –Agriculture Research Service. Thesis research was conducted at the USDA-ARS Forage and Range Research Laboratory in Logan, Utah, under the direction of Steve R. Larson. The major objectives of this initiative are to improve the availability of native plant materials and to provide the knowledge and technology necessary to restore diverse native plant communities across the Great Basin.

Catherine Mae Culumber

CONTENTS

	Page
ABSTRACT.....	ii
ACKNOWLEDGMENTS	iv
LIST OF TABLES.....	vii
LIST OF FIGURES	viii
CHAPTER	
1. INTRODUCTION	1
LITERATURE CITED	4
2. GENETIC ANALYSIS OF NORTH AMERICAN <i>LEYMUS</i> WILDRYES (POACEAE) AND OTHER <i>LEYMUS</i> TAXA	7
ABSTRACT.....	7
LITERATURE REVIEW	8
MATERIALS AND METHODS.....	14
Plant materials.....	14
DNA extractions	21
Evaluation of candidate cpDNA primers.....	21
Sequencing.....	21
Sequencing alignment and indel coding	22
Phylogenetic analysis.....	23
Divergence time estimates using a calibrated molecular clock	24
Genetic and geographic distance correlation	25
RESULTS	26
Informative value of candidate cpDNA primers.....	26
cpDNA sequences.....	26
cpDNA sequence AMOVA statistics.....	28
Patterns in cpDNA phylogenies.....	31
Simple molecular clock estimation	34
ITS sequences	34

	ITS sequences' AMOVA statistics	36
	ITS phylogenies	39
	Paired sites	44
	Genetic and geographic distance correlation	45
	DISCUSSION	45
	LITERATURE CITED	51
	APPENDIX A MORPHOLOGY	58
	APPENDIX B SEQUENCING PCR METHODS	59
	APPENDIX C CHARACTERIZATION OF CPDNA ALLELIC HAPLOTYPES	60
	APPENDIX D CHLOROPLAST DNA TOTAL AVERAGE NUMBER OF PAIRWISE DIFFERENCES	61
	APPENDIX E CHLOROPLAST DNA K2P AVERAGE NUMBER OF PAIRWISE DIFFERENCES	62
	APPENDIX F ITS TOTAL AVERAGE NUMBER OF PAIRWISE DIFFERENCES	63
	APPENDIX G ITS K2P AVERAGE NUMBER OF PAIRWISE DIFFERENCES	64
3.	GENETIC ANALYSIS OF <i>LEPIDIUM</i> (BRASSICACEAE) IN WESTERN NORTH AMERICA	65
	ABSTRACT	65
	INTRODUCTION	66
	LITERATURE REVIEW	69
	MATERIALS AND METHODS	73
	DNA isolation and barcoding	76
	Genetic analysis of chloroplast and ITS sequence variation	78
	RESULTS	80
	ITS DNA sequences	80
	Chloroplast DNA sequences	85
	Divergence time estimates using a calibrated molecular clock	89
	DISCUSSION	91
	LITERATURE CITED	94
4.	CONCLUSION	97
	LITERATURE CITED	100

LIST OF TABLES

Table		Page
2-1	Summary of <i>Leymus</i> accessions evaluated.....	15
2-2	Chloroplast DNA and ITS haplotype(s) and locality formation for 319 <i>Leymus</i> accessions	17
2-3	Pilot evaluation of sequence divergence among four intergenic spacers	26
2-4	Sequence characteristics of cpDNA and ITS sequences.....	27
3-1	List of <i>Lepidium</i> accessions included in the study	77
3-2	Frequency of 'N' numbers of individuals among <i>L. montanum</i> and <i>L. papilliferum</i> characterized by 19 ITS allelic haplotypes	84
3-3	Frequency of 'N' numbers of individuals among <i>L. montanum</i> and <i>L. papilliferum</i> characterized by 10 cpDNA allelic haplotypes.....	89

LIST OF FIGURES

Figure	Page
2-1	Accession distributions for six North American <i>Leymus</i> taxa..... 16
2-2	Heuristic parsimony analysis for 64 <i>Leymus</i> haplotypes and four other Triticeae taxa, based on the chloroplast <i>trnH-psbA</i> and <i>trnK-rps16</i> spacers 32
2-3	Neighbor-joining distance analysis for 64 <i>Leymus</i> haplotypes and four other Triticeae taxa based on the total number of differences (substitutions or indels) among the chloroplast <i>trnH-psbA</i> and <i>trnK-rps16</i> spacers..... 33
2-4	Neighbor-joining distance phylogeny based on the cpDNA Kimura two-parameter corrected average number of pairwise differences for 19 <i>Leymus</i> taxa 35
2-5	Heuristic parsimony analysis of 81 ITS haplotypes bootstrap values determined from 500 replicates 40
2-6	Phylogram constructed from neighbor-joining distance analysis among 19 <i>Leymus</i> taxa and two other Triticeae taxa, based on the total number of differences (substitutions or indels) among nuclear ribosomal DNA internal transcribed spacer (ITS) haplotypes..... 41
2-7	Neighbor-joining distance phylogeny based on the ITS Kimura's two-parameter corrected average number of pairwise differences for 19 <i>Leymus</i> taxa 43
3-1	a) Diagram of the cpDNA <i>trnL</i> intron and the <i>trnL-trnF</i> intergenic spacer region primers c through f entire b) internal transcribed spacer 18S-5.8S-26S..... 67
3-2	Distribution map of <i>Lepidium</i> accessions included in the study..... 75
3-3	UPGMA distance analysis among <i>Lepidium montanum</i> (LEMO), <i>L. papilliferum</i> (LEPA), and other North American <i>Lepidium</i> taxa based on the total number of differences (substitutions or indels) among nuclear ribosomal DNA internal transcribed spacer (ITS) haplotypes..... 81

3-4	Phylogeny of <i>Lepidium montanum</i> (LEMO) and <i>L. papilliferum</i> (LEPA) inferred from nuclear ribosomal DNA internal transcribed sequence (ITS) haplotypes, obtained using a heuristic parsimony search.....	83
3-5	UPGMA distance analysis among <i>Lepidium montanum</i> (LEMO), <i>L. papilliferum</i> (LEPA), and other North American <i>Lepidium</i> taxa based on the total number of differences (substitutions or indels) among the chloroplast <i>trnL</i> intron and <i>trnL-F</i> spacer DNA sequences.....	86
3-6	Phylogeny of <i>Lepidium montanum</i> (LEMO), <i>L. papilliferum</i> (LEPA), and other North American <i>Lepidium</i> taxa inferred from the chloroplast <i>trnL</i> intron and <i>trnL-F</i> intergenic spacer DNA sequences obtained using a heuristic parsimony search	88

CHAPTER 1

INTRODUCTION

DNA barcoding is a molecular technique developed in an attempt to identify and classify the 10-100 million organisms throughout the globe. The “Consortium for the Barcode of Life” (www.barcoding.si.edu) is a database of reference sequences (vouchers) by which unidentified specimens can be compared (Kress et al., 2005). Success has been attained in producing DNA barcodes in animals and algae using the subunit 1 of cytochrome c oxidase (COI) (Hebert et al., 2003). Efforts to barcode plants using the COI region have yielded highly invariant sequences in plants, suggesting plants have a much slower rate of evolution in the COI region. Two DNA regions have been proposed for barcoding plants for identification and sequence vouchering purposes. The most commonly sequenced regions include the internal transcribed spacer (ITS) region of the nuclear ribosomal cistron (18S-5.8S-26S) as well as the plastid chloroplast DNA (cpDNA) region.

Approximately 36 000 angiosperm ITS sequences are available in Genbank, making ITS the most frequently sequenced region of the plant nuclear genome (Baldwin, 1992, 1995; Hsiao et al., 1994, 1995; Álvarez and Wendel, 2003; Kress et al., 2005). Internal transcribed spacer sequences have also been used to discern fungal and bacterial phylogenies (Gardes and Bruns, 1993; Buchan et al., 2002). The accelerated rate at which the ITS region evolves may be sufficient to detect variation among species within a genus or among populations (White et al., 1990). The ITS locus is biparental inherited with intragenomic uniformity, that in many cases may eliminate confounding variation

within plants leaving only species and clade-specific character-state changes. The ITS has low functional constraint allowing for neutral evolution. Another advantage of the ITS region is that it can be amplified in two smaller fragments the 18S-5.8S and 5.8S-26S primers, using the 5.8S as a universal primer bridge, which has proven especially useful in degraded samples (Kress et al., 2005).

Non-coding cpDNA are usually maternally inherited, hemizygous, and evolve as non-recombining lineages. However, paternal and or biparental inheritance has been found in gymnosperms and several other taxa (Smith et al., 1986; Neale and Sederoff, 1989; Neale et al., 1989). It is hypothesized that the slowly evolving cpDNA genome will demonstrate greater molecular variation between species than within species. While some cpDNA regions provide enough information to infer relationships at the intrageneric level in some taxa, others have demonstrated the capacity to provide resolution at lower taxonomic levels (Baldwin, 1992; Soltis et al., 1992; Sang et al., 1997; Shaw et al., 2005). Increasing amounts of research are being conducted on the chloroplast genome, with up twenty-one non-coding cpDNA regions available for comparison among genera and species. Analyses of the predictive value of various cpDNA regions have been implemented to determine which sequences provide the greatest phylogenetic resolution. Universal PCR primers flanking noncoding sequences of the *trnL-trnL-trnF* region (Taberlet et al., 1991) rank among the first and most widely used regions in plant molecular systematics (Shaw et al., 2005). The plastid *trnH-psbA* intergenic spacer has also been found to have a high level of sequence divergence in the majority of genera tested and highest amplification among angiosperms tested (Aldrich et al., 1988; Sang et al., 1997; Tate and Simpson 2003; Kress et al., 2005).

The level of genetic variability produced by different markers can vary among different taxa. It has been suggested that greater phylogenetic resolution can be obtained when data from various regions of the plant genome are combined (Shaw et al., 2005). Inconsistencies between topologies constructed from different regions of the genome, may reflect lineage sorting, the retention of ancestral character states in the more slowly evolving uniparentally inherited cpDNA genome, ancestral hybridization, introgression, or polyploidization in one region independent from the another (Avice et al., 1987, Avice, 2001).

The primary objective of this research was to test the ability of DNA barcoding to distinguish plants to the species level. A secondary objective was to construct phylogenies from intraspecific and interspecific polymorphic information: Chapter II, “Genetic Analysis of North American *Leymus* Wildryes and other *Leymus* Taxa,” describes the analysis of DNA sequences for 19 *Leymus* species. *Leymus* is an ecologically important native forage grass used for large-scale rangeland revegetation and other agricultural conservation uses. Information about *Leymus* is needed to identify genetically diverse, geographically significant ecotypes of native species and varieties, appropriate for energy production, fire-rehabilitation and other large-scale revegetation needs in the western United States. Chapter III, “Genetic Analysis of *Lepidium* (Brassicaceae) in Western North America,” describes the comparison of DNA sequences from North American *Lepidium*. The question as to whether *L. papilliferum* merits species status, or if it should be considered a subspecies of *L. montanum* was brought to attention when the U.S. Fish and Wildlife Service proposed to *L. papilliferum* as an

endangered species. We hypothesized that the detection of unique polymorphisms would distinguish *L. papilliferum* from *L. montanum*.

LITERATURE CITED

- ALDRICH, J., B. W. CHERNEY, E. MERLIN, AND L. CHRISTOPHERSON. 1988. The role of insertions/deletions in the evolution of the intergenic region between *psbA* and *trnH* in the chloroplast genome. *Current Genetics* 14: 137-146.
- ÁLVAREZ, I., AND J. F. WENDEL. 2003. Ribosomal ITS sequences and plant phylogenetic inference. *Molecular Phylogenetics and Evolution* 29: 417-434.
- AVISE, J. C., J. ARNOLD, R. M. BALL, E. BERMINGHAM, T. LAMB, J. E. NEIGEL, C. A. REEB, AND N. C. SAUNDERS. 1987. Intraspecific phylogeography-the mitochondrial-DNA bridge between population genetics and systematics. *Annual Review of Ecology and Systematics* 18: 489-522.
- AVISE, J. C. 2001. Evolving genomic metaphors: A new look at the language of DNA. *Science* 294: 86-87.
- BALDWIN, B. G. 1992. Phylogenetic utility of the internal transcribed spacers of nuclear ribosomal DNA in plants: an example from the Compositae. *Molecular Phylogenetics and Evolution* 1:3-16.
- BALDWIN, B. G. 1995. The ITS region of nuclear ribosomal DNA- A valuable source of evidence on angiosperm phylogeny. *Annals of the Missouri Botanical Garden* 82 2: 247.
- BUCHAN, A., S. Y. NEWELL, J. L. MORETA, AND M. A. MORAN. 2002. Analysis of internal transcribed spacer (ITS) regions of RNA genes in fungal communities in southeastern U.S. salt marsh. *Microbial Ecology* 43: 329-340.
- GARDES, M., AND T. D. BRUNS. 1993. ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. *Molecular Ecology* 2:113-118.
- HEBERT, P. D. N., A. CYWINSKA, S. L. BALL, AND J. R. DEWAARD. 2003. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London Series B-Biological Sciences* 270: 96-99.

- KRESS J. W., K. J. WURDACK, E. A. ZIMMER, L. A. WEIGT, AND D. H. JANZEN. 2005. Use of DNA barcodes to identify flowering plants. *Proceeding of the National Academy of Sciences of the United States of America* 102: 8369-8374.
- HSIAO, C., N. J. CHATTERTON, K. H. ASAY, AND K. B. JENSEN. 1995. Phylogenetic relationships of monogenomic species of the wheat tribe, Triticeae (Poaceae), inferred from nuclear rDNA (internal transcribed spacer) sequences. *Genome* 38: 211-223.
- HSIAO, C., N. J. CHATTERTON, K. H. ASAY, AND K. B. JENSEN. 1994. Phylogenetic relationships of 10 grass species: and assessment of phylogenetic utility of the internal transcribed spacer region in nuclear ribosomal DNA in monocots. *Genome* 37: 112-120.
- NEALE D. B., AND, R. R. SEDEROFF. 1989. Paternal inheritance of chloroplast DNA and maternal inheritance of mitochondrial-DNA in Loblolly Pine. *Theoretical and Applied Genetics* 77: 212-216.
- NEALE D. B., K. A. MARSHALL, AND R. R. SEDEROFF. 1989. Chloroplast and mitochondrial-DNA are paternally inherited in *Sequoia sempervirens* (D. Don) Endl. *Proceedings of the National Academy of Sciences of the United States of America* 86: 9347-9349.
- SANG, T., D. J. CRAWFORD, AND T. F. STUESSY. 1997. Chloroplast DNA phylogeny, Reticulate Evolution, biogeography of *Paeonia* (Paeoniaceae). *American Journal of Botany* 84: 1120-1136.
- SHAW J., E. B. LICKEY, J. T. BECK, S. B. FARMER, W. S. LIU, J. MILLER, K. C. SIRIPUN, C. T. WINDER, E. E. SCHILLING, AND R. L. SMALL. 2005. The tortoise and the hare II: Relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *American Journal of Botany* 92: 142-166.
- SOLTIS, P., D. SOLTIS, AND J. DOYLE. 1992. *Molecular Systematics of Plants*. Routledge, Chapman and Hall Inc. New York, New York, USA.
- SMITH, S. E., E. T. BINGHAM, AND R. W. FULTON. 1986. Transmission of chlorophyll deficiencies in *Medicago sativa*. Evidence for biparental inheritance of plastids. *Journal of Heredity* 77: 35-38.
- TATE, J. A., AND B. B. SIMPSON. 2003. Paraphyly of *Tarasa* (Malvaceae) and diverse origins of the polyploid species. *Systematic Botany* 28: 723-737.
- TABERLET, P., L. GIELLY, G. PAUTOU, AND J. BOUVET. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* 17: 1105-1109.

WHITE, T. J., T. BURNS, S. LEE, AND J. TAYLOR. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In*: Innis, M., D. Gelfand, J. Sninsky, and T. White [eds.], PCR Protocols: A Guide to Methods and Applications, pp. 315-322. Academic Press, San Diego, California, USA.

CHAPTER 2

GENETIC ANALYSIS OF NORTH AMERICAN *LEYMUS*WILDRYES AND OTHER *LEYMUS* TAXA

ABSTRACT

An evaluation was conducted to determine the genetic variability among nine North American *Leymus* species collected in the western United States, and in British Columbia and Alberta, Canada. The purpose of this research was to acquire information about genetic relatedness within and among *Leymus* sp., and to test the efficacy of the proposed DNA sequence “barcoding” method for species identification. DNA barcodes were generated for North American, European, and Asian *Leymus* taxa, using the *trnH-psbA*, *trnK-rps16* regions of the chloroplast genome and the 18S-5.8S-26S nuclear ribosomal DNA internal transcribed spacer region of the nuclear genome. Three North American *Leymus* species, including *L. flavescens*, *L. innovatus*, and *L. mollis*, displayed loci unique to their species, in both chloroplast and internal transcribed spacer sequences. However, this specific set of DNA barcodes was insufficient to unambiguously identify individual plants in other *Leymus* taxa, including *L. cinereus* and *L. triticoides*, the dominant taxa in the sample set. Differences between North American *Leymus* and Eurasian outgroup taxa accounted for 84.53% of the total chloroplast DNA sequence variation. Chloroplast DNA phylogenies separated North American and Eurasian *Leymus* species into two distinct groups, with an estimated divergence time of $.65 \times 10^6$ to 2.3×10^6 million years ago. Eurasian *Leymus* sequences are more like *Psathyrostachys juncea*, which is one of the diploid ancestors of polyploid *Leymus*.

INTRODUCTION

The effort to increase the use of native plants for restoration was initiated in 2001 by the Great Basin Restoration Initiative in collaboration with the Agricultural Research Service (ARS), among several other federal agencies. The Great Basin Native Plant Selection and Increase Project (GBNSIP) was designed to improve the availability of 49 native species for restoration following disturbance or wildfire. Moreover, improved germplasm and cultivars for semi-arid public and private rangelands with the objective of improving germinability, seed production, plant establishment, persistence, forage and seed quality, and pest resistance. Specific goals of the GBNSIP includes: 1) the collection of representative germplasm throughout the distributional range of the species, 2) evaluation: including detection of the genetic patterns of variation among species, population structure, and functional and physiological traits of the species, 4) selection of materials based on characters such as seed production, seedling vigor, and plant vigor, 4) large-scale seed production, and finally, 5) application of the seed to the rehabilitated area.

The evaluation (step 2) of several Triticeae grass species, including bluebunch wheatgrass (*Pseudoroegneria spicata*) (Larson et al., 2000, 2004), western wheatgrass (*Pascopyrum smithii*) (Larson et al., 2003b), and squirreltail (*Elymus elymoides* and *Elymus multisetus*) (Jones et al., 2003; Larson et al., 2003a) was conducted to determine patterns of genetic diversity on the natural landscape and to infer the significance of population structure in these species. A similar research design was created to elucidate the level of genetic variation in the North American perennial grass, *Leymus* (wildrye).

Germplasm resources of high genetic diversity may be utilized for the development of rangeland resources as well as for providing information about allelic differences among species across floristic provinces.

Leymus is a close relative of wheat, barley, cultivated rye, and other Triticeae cereals that rank among the world's most important domesticated crop species. *Leymus* is an ecologically important native forage grass used for large-scale rangeland revegetation and other agricultural conservation uses. *Leymus* is known for its tolerance for high saline/alkaline conditions and its ability to produce abundant forage and quality habitat, particularly in the Great Basin region of the western U.S. and other semiarid temperate regions of the world. *Leymus* has been used for mine reclamation (Ferguson and Frischknecht, 1985), fire rehabilitation (Richards et al., 1998), and stabilization of other disturbed areas (Wasser, 1982). *Leymus* is also being evaluated to determine its use as an alternative source of biofuel energy in the western United States. Biofuels developed from perennial grasses could serve as an ideal energy source, as the production is cost-effective, renewable, and efficiently converted. Information is needed to help scientists and land managers identify, select, and develop germplasm sources appropriate for energy production, fire-rehabilitation and other large-scale revegetation needs in the western United States.

Leymus includes about 50 perennial grass species worldwide, from temperate regions of North America, South America, Europe, and Asia. *Leymus arenarius* is found in coastal areas of Europe, Asia, as well as North and South America. *Leymus angustus* and *L. chinensis* can be found across central and east Asia. Some North American taxa appear to have somewhat isolated distributions. *Leymus mollis* is found on coastal

shorelines and inland waters in Alaska and Asia. *Leymus ambiguus* grows in scattered locations throughout Colorado and New Mexico. *Leymus flavescens* is found along the Snake and Colombia River valleys in Idaho, Oregon, and Washington. *Leymus condensatus* inhabits the coast of California. Other North American taxa have continuous distributions across their range. *Leymus innovatus* occurs across Canada, south to Wyoming and South Dakota, while *L. cinereus*, *L. triticoides* and *L. salinus* demonstrate overlapping distributions with other *Leymus* species across western America (Bowden, 1957, 1964, 1967, Cronquist et al., 1977; Dewey, 1984; Barkworth and Atkins, 1984; Barkworth, 2007). In the western United States, *Leymus* species can be found at elevations between 762 and 2133 meters in saline/alkaline floodplains, alluvial fans, sagebrush semi-deserts, steppes, woodlands, and glaciated areas from the Colorado Plateau and the Great Basin to the southern, middle, and northern Rocky Mountains (Hickman, 1993).

Leymus is a segregate group of the Triticeae tribe, once assigned to the genus *Elymus* (Bentham, 1881; Hitchcock, 1935, 1951; Hitchcock et al., 1969), which first gained recognition as a species based on morphological characters by Pilger (1949) (see Appendix A for morphological description). The phylogenetic relatedness of all *Leymus* species is supported by evidence that all *Leymus* share the same basic allotetraploid combination of two genomes NsNsXmXm. This includes at least one set of chromosomes (NsNs) from *Psathyrostachys* (Zhang and Dvorak, 1991; Wang and Jensen, 1994; Wang et al., 1994; Ananthawat-Jonsson and Bodvarsdottir, 2001). *Psathyrostachys juncea* shows a high degree of similarity to at least one of the genomes of *L. cinereus* and *L. triticoides* (Ananthawat-Jonsson and Bodvarsdottir, 1998, 2001; Bodvarsdottir and

Anamthawat-Jonsson, 2003; Wu et al., 2003). Redinbaugh et al. (2000) found 14 to 15 nucleotide differences between *Psathyrostachys juncea* and two North American *Leymus* taxa for 753 bp *ndhF* chloroplast sequences. The other Xm genome has not been definitely identified, as cytogenetic and molecular data discounted the belief that it originated in *Thinopyrum* (Zhang and Dvorak, 1991; Wang and Jensen, 1994). The diploid ancestral species have not been conclusively identified, and it is possible that *Leymus* may not have monophyletic origins from the same two diploid ancestors. Moreover, octoploid and dodecaploid species presumably arise from within the allotetraploid *Leymus* gene pool. Most North American *Leymus* species are allotetraploid ($2n = 4x = 28$), yet in the northwestern portion of its range, e.g., British Columbia, Washington, Oregon, and Idaho, some *L. cinereus* are allooctoploid ($2n = 8x = 56$). On other continents, such as Asia, *L. angustus* is dodecaploid ($2n = 12x = 84$), emerging from interspecific hybrids or autoduplication within species (Dewey, 1972; Hole and Jensen, 1999).

Crossing *L. flavescens* with other North American species (*L. triticoides*, *L. cinereus*), as well as European (*L. arenarius*) with Asian tetraploids (*L. secalinus*, *L. racemosus*) produced sterile F1 hybrids, supporting the separate designation of some species (Hole and Jensen, 1999). Ribosomal gene mapping by Anamthawat-Jonsson and Bodvarsdottir (2001) concluded that Eurasian *L. arenarius* and *L. racemosus* are much more closely related to one another than to North American *L. mollis*, and the *L. arenarius* genome is likely to have evolved from the *L. racemosus* genome. Sterile hybrids between *L. mollis* and *L. arenarius* are common in regions of sympatric distribution (Barkworth and Atkins, 1984). On the other hand, fertile hybrids between *L.*

innovatus and *L. salinus* ssp., as well as between *L. cinereus* and *L. triticoides*, have produced seed, indicating weak breeding barriers among some *Leymus* species despite geographical barriers, distinct taxonomic assignments, and variability in chromosome numbers (Barkworth and Atkins, 1984; Hole and Jensen, 1999; Wu et al., 2003).

There are three single-origin releases of *L. cinereus* (Magnar, Trailhead, and Washoe) Germplasm and one of *L. triticoides* (Rio). Another release (Shoshone) was identified as *L. triticoides* when collected but later identified as *L. multicaulus* subsequent to its release by the United States Department of Agriculture (USDA)-Soil Conservation Service (SCS) Bridger Plant Materials Center in 1980 (Jones and Johnson, 1998; Barkworth and Jacobs, 2001). These materials were released because they possess various desirable characteristics, e.g., seed viability, soil-binding rhizomatous tillers, drought tolerance, heavy metal tolerance, low pH tolerance, and adaptability to a range of soil types. They have potential to improve forage, stabilization, cover, and conservation. Accessions of all five of these releases were included among the samples for genetic comparison. Magnar was increased in Pullman, WA from a collection made in British Columbia and was released in 1979 by the Aberdeen Plant Materials Center. Developed by selection of several vigorous types over several generations, it is adapted to the northwestern United States where precipitation normally exceeds 200 mm. Trailhead was collected in Roundup, MT and released by the Bridger Plants Material Center in 1991. It is adapted to the western Great Plains and the Intermountain Region. Due to its longevity and drought tolerance, Trailhead was increased for cultivation without selection. Both of these cultivars are adapted to weakly saline clay and loam soils and are somewhat tolerant of sandy substrates. Washoe Germplasm, released in 2002 by the Natural

Resources Conservation Service (NRCS) Bridger Plant Materials Center, was collected in the tailing of the Anaconda copper mine, a Superfund site, in Deerlodge, MT. Unlike the two cultivars, Washoe Germplasm possesses toleration for low pH soils with high levels of heavy metal contamination (Marty, 2002; Ogle et al., 2003). Shoshone was collected in Riverton, Wyoming. Released in 1980 from USDA-SCS Bridger Plant Materials Center, the seed was directly increased without selection. Rio was collected in 1984 in Kings Valley, California and was released by the USDA-SCS Lockeford Plant Materials Center. These accessions were included in the analysis to determine if there is a varying level of heterogeneity between increased and wild accessions.

The primary objective of this research was to test the ability of DNA barcoding to distinguish accessions to the species level. A pilot study was conducted to determine the predictive value of several candidate cpDNA genes. The *trnH-psbA*, *trnK-rps16* regions of the cpDNA region (Kress et al., 2005) and the ribosomal internal transcribed spacer (ITS) regions (Baldwin, 1992, 1995; Kress et al., 2005) were chosen to determine levels of genetic variation among *Leymus*. Studies (Kress et al., 2005; Shaw et al., 2005) suggest utility in the use of the internal transcribed species (ITS) and *trnH-psbA* intergenic spacers among other regions for species identification. According to Kress, *trnH-psbA* was one of three genes that successfully amplified eight genera and 19 species, with the highest level of divergence 1.81% compared to other plastid regions. The internal transcribed spacer regions had the highest between-species sequence divergence with a mean sequence divergence 2.81% across the five genera.

A secondary objective was to construct a phylogeny from intraspecific and interspecific polymorphic information in order to infer the phyletic or monophyletic

origins of North American *Leymus*. Studies suggest a combination of single genes may be sufficient to construct an accurate phylogeny if sequences display an adequate number of informative polymorphic sites (Kress et al., 2005; Shaw et al., 2005; Chase et al., 2005). We hypothesized that polymorphic data would also demonstrate a correlation between genetic distance and geographic distance within North American *L. cinereus* populations. The sampling design for the DNA study was constructed specifically to emphasize high-density sampling of as many sites as possible over a broad geographic region (Figure 1). It was hypothesized that such a design would enable the detection of genetic discontinuities or continuities within and between taxonomic designations.

MATERIALS AND METHODS

Plant materials- The *Leymus* evaluation included nine North American species collected in the western United States and British Columbia and Alberta, Canada. Evaluations included 250 *L. cinereus* accessions, 17 *L. triticoides* accessions, eight putative *L. cinereus* x *triticoides* hybrids, 21 accessions of seven other North American *Leymus* species, as well as 23 Eurasian *Leymus* (Table 2-1). Plant materials included 55 collections from the Great Basin Research Center, four accessions from the Natural Resource Conservation Service, four *Leymus cinereus* accessions collected by Berta Youtie, and 61 new Forage and Range Research Laboratory wildland seed collections from 2004. Source data are described in (Table 2-2) and in a map of the distribution area (Figure 2-1). A total of 319 plant accessions were included in the analysis. Seeds of each accession were collected and defined by their native-site origin. Of 296 native accessions, 203 are of wildland origin, 76 are increased from single-origin material or multiple-origin

Table 2-1. Summary of *Leymus* accessions evaluated.

<i>Leymus</i> North American Species	# of accessions	# Wild	# Increased	#Unknown
<i>L. cinereus</i>	250	176	69	5
<i>L. triticoides</i>	17	12	1	4
<i>L. cinereus</i> x <i>triticoides</i>	8	4	3	1
<i>L. salinus</i>	6	5	1	0
<i>L. condensatus</i>	2	0	0	2
<i>L. flavescent</i>	2	2	0	0
<i>L. ambiguus</i>	6	4	2	0
<i>L. innovatus</i>	2	0	0	2
<i>L. mojavenensis</i>	1	0	0	1
<i>L. mollis</i>	2	0	0	2
TOTAL	296	203	76	17

<i>Leymus</i> outgroup species	# of accessions	# Wild	# Increased	#Unknown
<i>L. multicaulis</i>	4	1	2*	1
<i>L. angustus</i>	2	0	0	2
<i>L. arenarius</i>	2	0	2	0
<i>L. chinensis</i>	2	0	0	2
<i>L. akmolinensis</i>	2	0	0	2
<i>L. racemosus</i>	2	1	0	1
<i>L. ramosus</i>	2	0	1	1
<i>L. secalinus</i>	5	0	0	5
<i>L. sabulosus</i>	2	0	0	2
TOTAL	23	2	5	16

North American and outgroups combined:	319	211	83	31
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**L. multicaulis* includes two SHOSHONE release accessions

material (five of which are USDA cultivars), and 17 are missing origin data. Coordinates for collection sites for wildland seed range from 37.9479°N, -114.40°W (*L. cinereus*) to 56.00°N, -117.00°W (*L. innovatus*) (Table 2-2 and Figure 2-1). To allow genetic comparisons between *Leymus* and other Triticeae grasses, 23 accessions of European or Asian descent, obtained from the National Plant Germplasm System, were included in the study. Seven additional accessions which served as controls for this experiment included Acc: 636 (*L. cinereus*) and Acc: 641 (*L. triticoides*) (the two parents of a controlled hybrid performed at the Agricultural Research Service, Logan, Utah); TC1 and TC2, two F1 hybrids (Wu et al., 2003); as well as *Psathyrostachys* and *Thinopyrum* accessions or Genbank submissions.

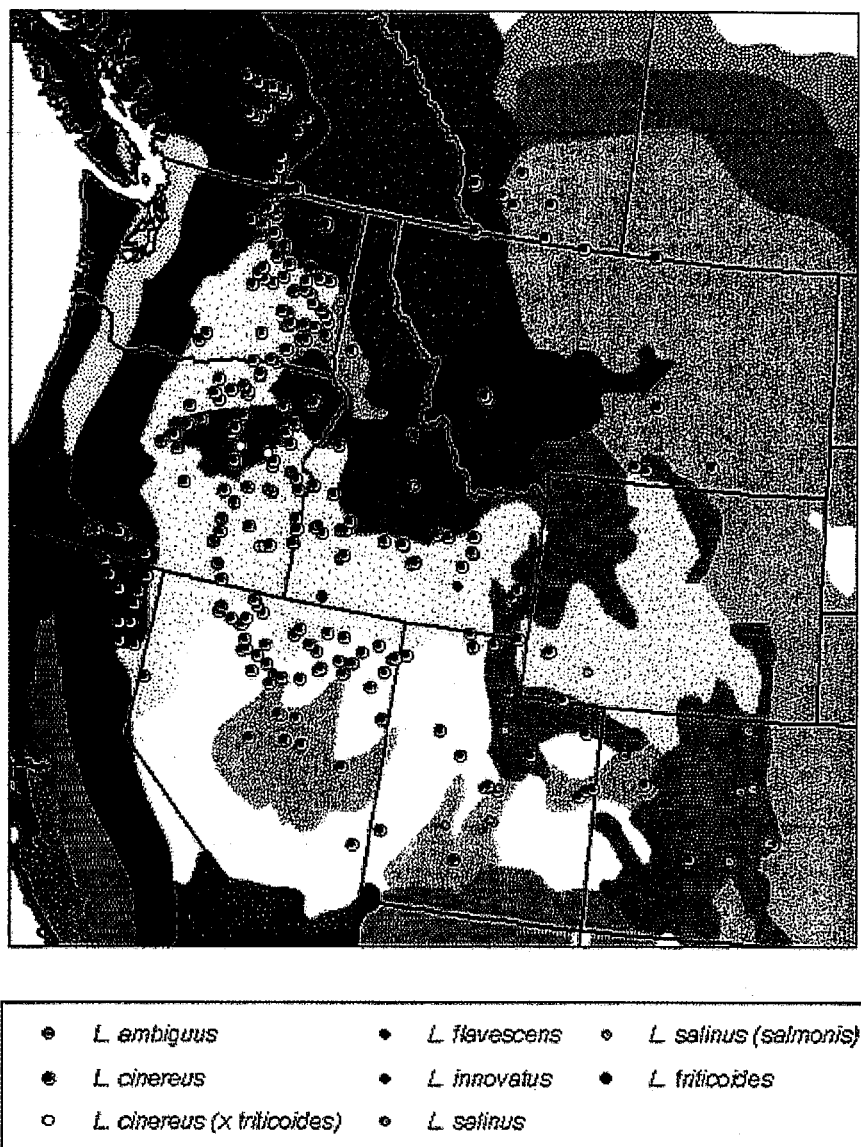


Figure 2-1. Accession distributions for six North American *Leymus* taxa. Taxa included in the study but not pictured in the map are: *L. condensatus*, *L. mollis*, and *L. innovatus*. Points are color coded according to species identification. Underlying colored regions represent Bailey's Ecosystem Provinces (Bailey and Cushwa, 1981), regions distinguished by vegetation composition.

Table 2-2 Chloroplast DNA and ITS Haplotype(s) and locality information for 319 *Leymus* accessions.

Haplotypes									
ITS	cpDNA	SAMPLE ID	Species	Accession	Origin	Location	Latitude	Longitude	
42,43	52	AKM_EU001	<i>L. akmolitensis</i>	DJ-3815	INC	Russia			
45,41	1	AKM_EU002	<i>L. akmolitensis</i>	P1531784	INC	Germany			
02,16	2	AMB_CO001	<i>L. ambiguus</i>	KJ85	WILD	Fremont, Co. Colo.	38.4604	-105.8482	
03,16	35	AMB_CO002	<i>L. ambiguus</i>	O-3330; P1531795	INC	El Paso Co., Colo.	38.7908	-104.8658	
16,16	3	AMB_CO003	<i>L. ambiguus</i>	KJ64; P1565019	WILD	El Paso Co., Colo.	38.9386	-104.8765	
16,21	4	AMB_CO005	<i>L. ambiguus</i>	KJ59 W6 10226	WILD	Gilpin, Co., Colo.	39.7840	-105.4301	
03,17	5	AMB_CO007	<i>L. ambiguus</i>	KJ54	WILD	Larimer Co., Colo.	40.7930	-105.6127	
16,17	6	AMB_CO009	<i>L. ambiguus</i>	P1531796	INC	Idaho Springs, Colo.	39.7403	-105.5283	
82,83	53	ANGUSTUS_EU1	<i>L. angustus</i>	D3664		MA-11-41-50 (original seed)			
84,85	53	ANGUSTUS_EU2	<i>L. angustus</i>	KJ150		zhi jiang			
40,39	65	ARENARIUS_EU1	<i>L. arenarius</i>	P1531800	INC	Lithuania			
40,39	56	ARENARIUS_EU2	<i>L. arenarius</i>	P1531801	INC	Norway			
44,44	7	CHINENSIS_EU1	<i>L. chinensis</i>	P1499514		Xilinhot			
44,44	36	CHINENSIS_EU2	<i>L. chinensis</i>	P1499515		Xilinhot			
03,21	40	CIN_AB001	<i>L. cinereus</i>	Acc:314	INC	Cypress Hills, AB	49.0203	-109.0225	
56,03	41	CIN_BC001	<i>L. cinereus</i>	Acc:319	INC	Kamloops, BC	51.18667	-120.7500	
12,01	42	CIN_BC003	<i>L. cinereus</i>	T-1073	WILD	Croville / Nighthawk, BC	48.9670	-119.4820	
03,18	40	CIN_BC004	<i>L. cinereus</i>	Writing on Stone-1994	WILD	Writing on Stone, BC	49.0000	-111.0000	
03,27	41	CIN_BC005	<i>L. cinereus</i>	T-1071	WILD	Bridlewell, BC	49.0090	-119.2188	
55,33	41	CIN_BC006	<i>L. cinereus</i>	T-1072	WILD	Rock Creek, BC	49.0683	-119.0367	
03,27	41	CIN_BC007	<i>L. cinereus</i>	T-1050	WILD	Cawston, BC	49.1690	-119.7422	
03,26	42	CIN_BC008	<i>L. cinereus</i>	T-1070	WILD	Penttison, BC	49.3737	-120.7600	
27,24	41	CIN_BC009	<i>L. cinereus</i>	T-1069	WILD	Summerland, BC	49.5725	-119.6378	
03,21	40	CIN_BC010	<i>L. cinereus</i>	P1 McLeod - 1994	WILD	Fort McLeod, BC	49.7200	-113.4425	
03,03	30	CIN_BC011	<i>L. cinereus</i>	Bob Creek - 1994	WILD	Bob Creek, BC	49.8572	-114.2347	
03,03	41	CIN_BC012	<i>L. cinereus</i>	T-1068	WILD	Kalamalka Lake, BC	50.0745	-119.3922	
03,01	42	CIN_BC013	<i>L. cinereus</i>	T-1064	WILD	Nicola, BC	50.1430	-120.6366	
03,24	42	CIN_BC014	<i>L. cinereus</i>	T-1063	WILD	Nicola, BC	50.1563	-120.6750	
03,03	30	CIN_BC015	<i>L. cinereus</i>	Slavely - 1994	WILD	Slavely, BC	50.1833	-113.0000	
12,32	29	CIN_BC016	<i>L. cinereus</i>	T-1062	WILD	NW Merritt, BC	50.2940	-120.8408	
12,32	41	CIN_BC017	<i>L. cinereus</i>	T-1067	WILD	Vernon, BC	50.3203	-119.2403	
03,03	42	CIN_BC018	<i>L. cinereus</i>	T-1065	WILD	N Nicola, BC	50.3537	-120.4013	
03,01	42	CIN_BC019	<i>L. cinereus</i>	T-1066	WILD	Monte Creek, BC	50.6417	-119.9252	
03,27	29	CIN_BC020	<i>L. cinereus</i>	T-1055	WILD	E Kamloops, BC	50.6563	-120.1090	
03,03	41	CIN_BC021	<i>L. cinereus</i>	T-1056	WILD	E Kamloops, BC	50.6583	-120.0915	
12,32	41	CIN_BC022	<i>L. cinereus</i>	T-1058	WILD	Kamloops Lake, BC	50.7027	-120.6023	
27,24	6	CIN_BC023	<i>L. cinereus</i>	T-1054	WILD	N Kamloops, BC	50.7037	-120.4393	
03,26	42	CIN_BC024	<i>L. cinereus</i>	T-1052	WILD	Paul Lake, BC	50.7075	-120.3015	
03,27	41	CIN_BC025	<i>L. cinereus</i>	T-1057	WILD	Kamloops, BC	50.7075	-120.4247	
03,01	41	CIN_BC026	<i>L. cinereus</i>	T-1059	WILD	S Savona, BC	50.7185	-120.6385	
03,01	42	CIN_BC027	<i>L. cinereus</i>	T-1051	WILD	Paul Lake, BC	50.7307	-120.6688	
12,32	41	CIN_BC028	<i>L. cinereus</i>	T-1060	WILD	S Savona, BC	50.7388	-120.6227	
03,27	41	CIN_BC029	<i>L. cinereus</i>	T-1053	WILD	N Kamloops, BC	50.7392	-120.4050	
03,01	41	CIN_BC030	<i>L. cinereus</i>	T-1061	WILD	Savona / Cache Creek, BC	50.7847	-121.1993	
03,21	43	CIN_BC031	<i>L. cinereus</i>	Maycroft - 1994	WILD	Maycroft, BC	49.0833	-112.0833	
03,03	43	CIN_BC032	<i>L. cinereus</i>	Porkys	WILD	Porkys, BC	49.0169	-114.0900	
03,19	40	CIN_BC033	<i>L. cinereus</i>	Oldman Dam - 1994	WILD	Oldman Dam, BC	49.7055	-112.0833	
03,05	41	CIN_CA001	<i>L. cinereus</i>	T-1535	WILD	S Ravendale, Calif.	40.5840	-120.2520	
03,03	41	CIN_CA002	<i>L. cinereus</i>	T-1531	WILD	N Willow Creek, Calif.	40.5866	-120.6382	
03,03	44	CIN_CA003	<i>L. cinereus</i>	T-1530	WILD	N Susanville, Calif.	40.9052	-120.7415	
03,03	9	CIN_CA004	<i>L. cinereus</i>	T-1536	WILD	N Terno, Calif.	40.9685	-120.5012	
03,03	42	CIN_CA005	<i>L. cinereus</i>	T-1537	WILD	N Ukely, Calif.	41.2753	-120.5018	
29,03	45	CIN_CA006	<i>L. cinereus</i>	T-1526	WILD	NW Canby, Calif.	41.4718	-120.8407	
03,03	44	CIN_CA007	<i>L. cinereus</i>	T-1538	WILD	Alturas, Calif.	41.5227	-120.4702	
03,03	45	CIN_CA008	<i>L. cinereus</i>	T-1524	WILD	SE Tulelake, Calif.	41.6700	-121.2552	
03,03	10	CIN_CA009	<i>L. cinereus</i>	T-1540	WILD	Goose Lake, Calif.	41.8217	-120.3512	
03,03	42	CIN_CA010	<i>L. cinereus</i>	T-1523	WILD	Tulelake, Calif.	41.9212	-121.4358	
03,21	43	CIN_CO001	<i>L. cinereus</i>	T-933	INC	Parlin, Colo.	38.4357	-106.7575	
03,03	39	CIN_CO002	<i>L. cinereus</i>	T-938	INC	Rio Blanco Co., Colo.	38.6836	-107.9183	
03,03	40	CIN_ID001	<i>L. cinereus</i>	Acc:386	INC	Soda Springs, Idaho	42.7146	-111.5608	
03,21	40	CIN_ID003	<i>L. cinereus</i>	T-1645	WILD	Owyhee Co., Idaho/Oreg border	42.9417	-115.7501	
03,10	44	CIN_ID004	<i>L. cinereus</i>	Acc:384	INC	Richfield, Idaho	43.0167	-114.1332	
88,58	40	CIN_ID005	<i>L. cinereus</i>	U104-01	WILD	Lincoln Co., Idaho	43.0467	-114.0336	
03,10	40	CIN_ID006	<i>L. cinereus</i>	U98-01	WILD	Bingham Co., Coffee Point, Idaho	43.1259	-112.8603	
03,67	40	CIN_ID007	<i>L. cinereus</i>	U105-01	WILD	Owyhee Co., Oreg	43.2478	-117.0262	
03,67	41	CIN_ID008	<i>L. cinereus</i>	U106-01	WILD	Owyhee Co., Idaho/Oreg border	43.2476	-117.0262	
03,21	43	CIN_ID009	<i>L. cinereus</i>	U110-01	WILD	Swan Falls Dam, Ada Co. Idaho	43.2657	-116.3937	
03,21	40	CIN_ID010	<i>L. cinereus</i>	T-2	WILD	Magic Reservoir, Blaine Co. Idaho	43.2167	-114.3533	
03,03	43	CIN_ID011	<i>L. cinereus</i>	T-4	WILD	Fairfield/Corral, Camas Co., Idaho	43.3167	-114.0567	
06,21	41	CIN_ID012	<i>L. cinereus</i>	U103-01	WILD	Mountain Landing, Blaine Co., Idaho	43.3266	-114.4040	
03,03	11	CIN_ID013	<i>L. cinereus</i>	T-1648	WILD	Owyhee Co., Idaho	43.3349	-116.8069	
03,25	41	CIN_ID014	<i>L. cinereus</i>	U100-01	WILD	Hwy 26 MM 280-281, Bingham Co. Idaho	43.3623	-112.6486	
03,03	40	CIN_ID015	<i>L. cinereus</i>	Acc:390	WILD	Ada Co. Idaho	43.3687	-116.0000	
26,20	40	CIN_ID016	<i>L. cinereus</i>	T-1212	WILD	SE Mayfield, Elmore Co. Idaho	43.3922	-115.8565	
03,03	40	CIN_ID017	<i>L. cinereus</i>	U101-01	WILD	Hwy 26 MM 289, Bingham Co. Idaho	43.4987	-112.7100	
03,03	40	CIN_ID018	<i>L. cinereus</i>	Acc:401	INC	South Fork Boise, Elmore Co. Idaho	43.5900	-115.8000	
03,03	43	CIN_ID019	<i>L. cinereus</i>	U99-01	WILD	Craters of the Moon Butte Co., Idaho	43.8057	-113.3255	
03,03	46	CIN_ID020	<i>L. cinereus</i>	Acc:297	WILD	Arco, Butte Co., Idaho	43.6187	-113.6000	
03,10	40	CIN_ID021	<i>L. cinereus</i>	Acc:71	WILD	Arco, Butte Co., Idaho	43.6667	-112.7900	
20,26	40	CIN_ID022	<i>L. cinereus</i>	T-23	WILD	New Plymouth, Gem Co. Idaho	43.6687	-116.3167	
03,03	41	CIN_ID023	<i>L. cinereus</i>	U107-01	WILD	Fort Wilson Rd Jct, Payette Co. Idaho	44.0331	-116.8486	
03,10	40	CIN_ID024	<i>L. cinereus</i>	U106-01	WILD	Payette Ft Wilson Rd, Payette Co., Idaho	44.0331	-116.8486	
03,03	40	CIN_ID025	<i>L. cinereus</i>	T-1648	WILD	Adams Co., Idaho	44.8351	-116.4219	
25,35	40	CIN_ID026	<i>L. cinereus</i>	T-970	INC	Orofino, Clearwater Co. Idaho	46.5202	-116.6183	
03,25	42	CIN_ID027	<i>L. cinereus</i>	Acc:317	?	Aberdeen, Bingham Co., Idaho			

Table 2-2. Chloroplast DNA and ITS Haplotype(s) and locality information for 319 *Leymus* accessions. (continued)

Haplotypes		Sample ID	Species	Accession	Origin	Location	Latitude	Longitude
ITS	cpDNA							
03.29	40	CIN_MT001	<i>L. cinereus</i>	8083352	WILD	Bighorn Co., Mont.	45.2391	-108.5282
03.31	34	CIN_MT003	<i>L. cinereus</i>	Acc:387	WILD	Bastry	45.4000	-107.0000
03.03	40	CIN_MT007	<i>L. cinereus</i>	PI472331	WILD	Mussellshell Co., Mont.	46.3882	-108.5409
03.03	39	CIN_MT008	<i>L. cinereus</i>	Acc:70	INC	Roundup, Mont.		
03.07	43	CIN_NV001	<i>L. cinereus</i>	Ely	WILD	Ely, Whitepine Co., Neva.	39.2618	-114.9308
03.03	44	CIN_NV002	<i>L. cinereus</i>	Gold Venture Rd.	WILD	Gold Venture Road, Lander Co., Neva.	39.4430	-117.1761
03.03	43	CIN_NV004	<i>L. cinereus</i>	T-46	WILD	Eureka Co., Neva.	39.4967	-115.9500
03.20	44	CIN_NV007	<i>L. cinereus</i>	Gord.Mall.Austin	WILD	Gordon Mailbox Austin, Lander Co., Neva.	39.4748	-117.0022
03.03	40	CIN_NV005	<i>L. cinereus</i>	B19-D1	WILD	Eureka Co., Neva.	39.5105	-115.9598
03.03	44	CIN_NV009	<i>L. cinereus</i>	Bartline Ranch	WILD	Bartline Ranch, Eureka Co., Neva.	39.5276	-116.3352
03.03	46	CIN_NV010	<i>L. cinereus</i>	U95-01	WILD	Pioche, Lincoln Co., Neva.	37.8478	-114.4006
03.20	41	CIN_NV011	<i>L. cinereus</i>	U113-02	WILD	Beaman Ranch, Eureka Co., Neva.	39.9488	-116.5475
03.03	41	CIN_NV012	<i>L. cinereus</i>	T-44	WILD	Eureka Co., Neva.	38.9500	-116.1667
03.03	12	CIN_NV013	<i>L. cinereus</i>	U97-01	WILD	Gund Ranch, Lander Co., Neva.	39.9561	-116.6052
03.03	44	CIN_NV014	<i>L. cinereus</i>	B16-01	WILD	Fish Springs Road, Washoe Co., Neva.	40.0654	-116.8810
03.21	40	CIN_NV015	<i>L. cinereus</i>	U96-02	INC	Sugarloaf South, Elko Co., Neva.	40.2117	-114.1985
03.03	41	CIN_NV016	<i>L. cinereus</i>	Acc:683	WILD	Crescent Valley, Neva.	40.4667	-116.9500
03.03	40	CIN_NV017	<i>L. cinereus</i>	U91-01	WILD	Sturdy Enclosure, Lander Co., Neva.	40.5538	-116.6654
06.21	41	CIN_NV018	<i>L. cinereus</i>	U90-01	INC	West of Water Canyon, Lander Co., Neva.	40.6140	-116.5809
03.03	44	CIN_NV019	<i>L. cinereus</i>	Acc:397	WILD	Battle Mountain, Humboldt Co., Neva.	40.6167	-117.4333
03.03	41	CIN_NV020	<i>L. cinereus</i>	BK-45	WILD	Carlin West Quad, Eureka Co., Neva.	40.5361	-116.2436
20.66	36	CIN_NV020C	<i>L. cinereus</i>	BK-45	WILD	Carlin West Quad, Eureka Co., Neva.	40.5361	-116.2436
03.03	42	CIN_NV021	<i>L. cinereus</i>	BK-44	WILD	Carlin West Quad, Eureka Co., Neva.	40.6383	-116.2439
03.03	42	CIN_NV021C	<i>L. cinereus</i>	BK-44	WILD	Carlin West Quad, Eureka Co., Neva.	40.6383	-116.2439
03.20	30	CIN_NV022	<i>L. cinereus</i>	U81-02	INC	Pequop Tunnel, Elko Co., Neva.	40.7315	-114.5726
03.25	41	CIN_NV023	<i>L. cinereus</i>	Acc:396	INC	Mole, Humboldt Co., Neva.	40.7633	-117.0633
03.03	41	CIN_NV025	<i>L. cinereus</i>	U82-02	WILD	Eureka, Eureka Co., Neva.	39.5105	-115.9598
03.03	44	CIN_NV027	<i>L. cinereus</i>	T-54	WILD	Secel Pass, Elko Co., Neva.	40.9000	-115.2833
26.20	46	CIN_NV028	<i>L. cinereus</i>	T-55	INC	Secel Pass, Elko Co., Neva.	40.9000	-115.2667
03.08	41	CIN_NV030	<i>L. cinereus</i>	BK-39	WILD	Iron Point Quad, Humboldt Co., Neva.	40.9025	-117.3663
26.21	44	CIN_NV031	<i>L. cinereus</i>	Acc:369	INC	Winnemucca, Humboldt Co., Neva.	40.6500	-117.7500
03.20	13	CIN_NV033	<i>L. cinereus</i>	U82-02	WILD	Toano, Elko Co., Neva.	41.0564	-114.3285
03.03	41	CIN_NV034	<i>L. cinereus</i>	T-30	WILD	Deeth, Elko Co., Neva.	41.0687	-115.2833
03.03	41	CIN_NV035	<i>L. cinereus</i>	Acc:385	WILD	Wells, Elko Co., Neva.	41.1000	-115.1000
06.09	41	CIN_NV036	<i>L. cinereus</i>	Acc:389; PI563865	INC	Montello, Elko Co., Neva.	41.1000	-115.4333
03.03	44	CIN_NV038	<i>L. cinereus</i>	BK-28	WILD	Dry Hills Flat Quad, Humboldt Co., Neva.	41.1253	-117.2379
03.19	40	CIN_NV039	<i>L. cinereus</i>	U83-02	WILD	Humboldt Co., Sand Pass	41.1372	-117.7330
03.03	41	CIN_NV040	<i>L. cinereus</i>	T-35	WILD	Lone Mountain, Humboldt Co., Neva.	41.2600	-115.9833
03.03	14	CIN_NV041	<i>L. cinereus</i>	T-37	WILD	Tuscarora, Elko Co., Neva.	41.2833	-116.1833
03.08	41	CIN_NV043	<i>L. cinereus</i>	U84-02	WILD	N.E. Montello, Elko Co., Neva.	41.3088	-114.1173
03.03	41	CIN_NV044	<i>L. cinereus</i>	BK-3	WILD	Elko Co., Neva.	41.3168	-114.9361
03.08	34	CIN_NV045	<i>L. cinereus</i>	Acc:321	INC	Montello, Elko Co., Neva.	41.3167	-114.1167
03.03	30	CIN_NV046	<i>L. cinereus</i>	BK-33	WILD	Gallagher Flat Quad, Humboldt Co., Neva.	41.3568	-117.9378
03.03	30	CIN_NV047	<i>L. cinereus</i>	U85-02	WILD	Quinn River Valley, Humboldt Co., Neva.	41.4021	-117.8544
03.20	41	CIN_NV048	<i>L. cinereus</i>	BK-26	WILD	Soldier Cap Quad, Humboldt Co., Neva.	41.4025	-116.9393
03.03	41	CIN_NV050	<i>L. cinereus</i>	U87-02	WILD	Thousand Spring, Elko Co., Neva.	41.4890	-114.5623
03.20	41	CIN_NV051	<i>L. cinereus</i>	T-1701	WILD	S Orada, Humboldt Co., Neva.	41.5080	-117.7919
06.09	16	CIN_NV052	<i>L. cinereus</i>	Acc:333	INC	Halleck, Elko Co., Neva.	41.5167	-115.4167
03.03	38	CIN_NV053	<i>L. cinereus</i>	U94-01	WILD	Owyhee Desert, Elko Co., Neva.	41.5206	-116.5601
03.03	16	CIN_NV054	<i>L. cinereus</i>	BK-34	WILD	Quinn River Valley, Humboldt Co., Neva.	41.5263	-118.4772
03.03	40	CIN_NV055	<i>L. cinereus</i>	U115-02	WILD	Hwy 226, Elko Co., Neva.	41.5361	-115.8364
03.03	17	CIN_NV056	<i>L. cinereus</i>	T-1658	WILD	Jct. Quinn River RD x Hwy 140, Humboldt Co., Neva.	41.5520	-118.4317
03.03	47	CIN_NV058C	<i>L. cinereus</i>	T-1658	WILD	Jct. Quinn River RD x Hwy 140, Humboldt Co., Neva.	41.5520	-118.4317
03.03	41	CIN_NV056	<i>L. cinereus</i>	Acc:381	WILD	Wells, Elko Co., Neva.	41.7500	-112.3333
03.21	41	CIN_NV059	<i>L. cinereus</i>	U112-02	WILD	Buckskin Road, Humboldt Co., Neva.	41.7703	-117.6970
20.66	41	CIN_NV060	<i>L. cinereus</i>	BK-30	WILD	Humboldt Co., Neva	41.8438	-117.6526
03.03	44	CIN_NV063	<i>L. cinereus</i>	(TR57) Nevada	WILD	Neva		
03.03	44	CIN_NV064	<i>L. cinereus</i>	U80-01	WILD	Mt. Dutton, Garfield Co., Utah	37.9500	-112.6872
03.26	44	CIN_OR001	<i>L. cinereus</i>	T-415	INC	N Dorio, Harney Co., Oreg.	42.0867	-118.6000
03.03	37	CIN_OR003	<i>L. cinereus</i>	T-1696	WILD	5.6 Mi NW Fields, Oreg.	42.3267	-118.7694
03.03	37	CIN_OR003C	<i>L. cinereus</i>	T-1696	WILD	5.6 Mi NW Fields, Oreg.	42.3267	-118.7694
03.27	44	CIN_OR005	<i>L. cinereus</i>	T-1520	WILD	Upper Klamath Lake, Oreg.	42.3533	-121.4215
03.03	48	CIN_OR006	<i>L. cinereus</i>	T-1517	WILD	W Bly, Oreg.	42.4086	-121.0593
03.01	44	CIN_OR007	<i>L. cinereus</i>	T-1518	WILD	Beatty, Oreg.	42.4372	-121.2745
03.34	18	CIN_OR008	<i>L. cinereus</i>	T-1694	WILD	14 Mi. S French Glen, Oreg.	42.6793	-118.9980
03.03	49	CIN_OR008C	<i>L. cinereus</i>	T-1694	WILD	14 Mi. S French Glen, Oreg.	42.6793	-118.9980
03.03	40	CIN_OR011	<i>L. cinereus</i>	T-1653	WILD	Rome, Malheur, Oreg.	42.8413	-117.6255
03.03	44	CIN_OR011C	<i>L. cinereus</i>	T-1653	WILD	Rome, Malheur, Oreg.	42.8413	-117.6255
06.03	41	CIN_OR012	<i>L. cinereus</i>	T-418	INC	French Glen, Oreg.	42.8533	-118.4833
03.03	40	CIN_OR013	<i>L. cinereus</i>	T-1650	WILD	Malheur Co., Oreg.	42.9761	-117.0580
03.03	41	CIN_OR014	<i>L. cinereus</i>	T-1659	WILD	Malheur Co., Oreg.	43.0605	-118.2058
03.03	44	CIN_OR015	<i>L. cinereus</i>	T-1652	WILD	22.6 m S French Glen, Oreg.	43.1178	-118.9139
03.03	19	CIN_OR016	<i>L. cinereus</i>	U114-02	WILD	Hwy 95 MM 3, Malheur Co., Oreg.	43.2122	-117.0466
03.01	20	CIN_OR017	<i>L. cinereus</i>	T-1649	WILD	Malheur Co., Oreg.	43.4078	-117.1185
03.03	36	CIN_OR018	<i>L. cinereus</i>	T-1660	WILD	Craneflawen, Harney Co., Oreg.	43.4394	-118.6046
03.03	40	CIN_OR019	<i>L. cinereus</i>	T-1691	WILD	S Burns, Oreg.	43.5650	-119.0216
03.27	41	CIN_OR020	<i>L. cinereus</i>	T-1669	WILD	SE Hampton, Oreg.	43.5856	-120.0219
03.34	41	CIN_OR021	<i>L. cinereus</i>	T-1662	WILD	East of Burns, Oreg.	43.6305	-116.6032
03.03	41	CIN_OR022	<i>L. cinereus</i>	T-1663	WILD	H. 20 and Stinking Water Cr., 33 Mi. E Burns, Oreg.	43.7637	-118.4545
03.03	21	CIN_OR023	<i>L. cinereus</i>	T-1664	WILD	Malheur River Canyon, Malheur Co., Oreg.	43.7870	-117.8471
03.25	22	CIN_OR023C	<i>L. cinereus</i>	T-1664	WILD	Malheur River Canyon, Malheur Co., Oreg.	43.7870	-117.8471
03.03	42	CIN_OR024	<i>L. cinereus</i>	U108-01	WILD	Keeney Pass, Malheur Co., Oreg.	43.9100	-117.1768
03.03	41	CIN_OR025	<i>L. cinereus</i>	T-1667	WILD	8.2 M NW Vale, Oreg.	44.0940	-117.3316
03.03	41	CIN_OR026	<i>L. cinereus</i>	Deschutes Basin	WILD	Deschutes Basin, Crook Co., Oreg.	44.1253	-120.3744
03.03	41	CIN_OR027	<i>L. cinereus</i>	T-1676	WILD	1.6 M N Ukiah, Oreg.	44.1611	-118.9527

Table 2-2. Chloroplast DNA and ITS Haplotype(s), and locality information for 319 *Leymus* accessions. (continued)

Haplotypes		Sample ID	Species	Accession	Origin	Location	Latitude	Longitude
ITS	cpDNA							
03,01	48	CIN_OR028	<i>L. cinereus</i>	T-1688	WILD	9.6 M S Prineville, Oreg.	44.1792	-120.8470
03,25	41	CIN_OR029	<i>L. cinereus</i>	Acc-370	INC	Ironsides, Oreg.	44.2333	-117.9987
03,03	42	CIN_OR030	<i>L. cinereus</i>	T-1687	WILD	10 M E Prineville, Oreg.	44.3080	-120.6413
03,03	30	CIN_OR031	<i>L. cinereus</i>	T-1669	WILD	E edge of Ironsides, Oreg.	44.3236	-117.9434
03,01	41	CIN_OR035	<i>L. cinereus</i>	T-1684	WILD	7.3 Mi, W of 28/207 Jct, Wheeler Co. Oreg.	44.5736	-120.2889
03,03	30	CIN_OR036	<i>L. cinereus</i>	T-1672	WILD	Grant Co. Oreg.	44.6310	-119.1485
03,25	24	CIN_OR037	<i>L. cinereus</i>	T-1011	INC	Pleasant Valley, Oreg.	44.6732	-117.6410
03,20	41	CIN_OR039	<i>L. cinereus</i>	T-1674	WILD	1.0 Mi N. Long Creek, Oreg.	44.7281	-119.1042
03,03	41	CIN_OR040	<i>L. cinereus</i>	T-1630	WILD	Wheeler Co., Oreg.	44.7373	-120.0051
03,27	40	CIN_OR041	<i>L. cinereus</i>	T-1010	INC	N Baker, Oreg.	44.8572	-117.7862
03,09	41	CIN_OR042	<i>L. cinereus</i>	John Day Basin	WILD	John Day Basin, Wheeler Co., Oreg.	44.9275	-120.3700
03,34	42	CIN_OR043	<i>L. cinereus</i>	T-1875	WILD	.2 Mi S of Dale, Oreg.	44.9899	-118.9512
03,27	48	CIN_OR044	<i>L. cinereus</i>	T-1009	INC	Medical Springs, Oreg.	45.0372	-117.6403
03,01	49	CIN_OR045	<i>L. cinereus</i>	T-1882	WILD	2.8 m Shardman, Morrow Co., Oreg.	45.1359	-119.6716
03,31	40	CIN_OR046	<i>L. cinereus</i>	T-1626	WILD	Hepner/Condon, Oreg.	45.2558	-119.8290
03,27	43	CIN_OR047	<i>L. cinereus</i>	T-1007	INC	La Grande, Oreg.	45.2865	-118.0347
03,03	25	CIN_OR048	<i>L. cinereus</i>	T-1677	WILD	16.3 m N Ukiah, Oreg.	45.3668	-118.9982
30,01	42	CIN_OR049	<i>L. cinereus</i>	T-1680	WILD	5.9 m E Heppner, Oreg.	45.3656	-119.4242
03,27	40	CIN_OR051	<i>L. cinereus</i>	T-1006	INC	W Enterprise, Oreg.	45.4410	-117.3507
03,03	42	CIN_OR052	<i>L. cinereus</i>	T-1679	WILD	Heppner/Pilot Rock, Umatilla Co., Oreg.	45.4911	-119.1315
03,10	44	CIN_OR054	<i>L. cinereus</i>	T-1681	WILD	SE lone, Morrow Co., Oreg.	45.4991	-119.7911
03,34	41	CIN_OR056	<i>L. cinereus</i>	T-19	WILD	Adams, Oreg.	45.7687	-118.7833
03,27	41	CIN_OR057	<i>L. cinereus</i>	T-1001	INC	Umatilla, Oreg.	45.9547	-119.0366
03,01	42	CIN_OR058	<i>L. cinereus</i>	Grande Ronde Basin	WILD			
03,03	42	CIN_UO002	<i>L. cinereus</i>	Acc-306	?			
66,88	40	CIN_UO004	<i>L. cinereus</i>	Acc-99	INC			
03,20	40	CIN_UT001	<i>L. cinereus</i>	U80-01	WILD	Mt. Dutton, Garfield Co., Utah	37.9500	-112.0872
03,20	41	CIN_UT002	<i>L. cinereus</i>	U87-01	WILD	Indian Peaks Cabin, Beaver Co., Utah	38.2736	-113.8145
03,27	42	CIN_UT003	<i>L. cinereus</i>	U78-01	WILD	Pretty Valley, Sanpete Co., Utah	39.2970	-111.5767
65,03	42	CIN_UT005	<i>L. cinereus</i>	U79-01	WILD	Majors Flat, Sanpete Co., Utah	39.3348	-111.5210
03,21	43	CIN_UT006	<i>L. cinereus</i>	U73-01	WILD	Hay Canyon, Grand Co., Utah	39.3820	-109.3896
27,24	43	CIN_UT007	<i>L. cinereus</i>	U75-01	WILD	South Canyon, Uintah Co., Utah	39.4773	-109.2385
03,67	40	CIN_UT008	<i>L. cinereus</i>	U76-01	WILD	Dick Canyon, Uintah Co., Utah	39.5127	-109.0932
03,03	41	CIN_UT009	<i>L. cinereus</i>	Acc-371	INC	Howell, Utah	39.6167	-110.8657
03,21	43	CIN_UT010	<i>L. cinereus</i>	U72-01	WILD	West Tintic Valley, Juab Co., Utah	39.7997	-112.2651
03,10	43	CIN_UT011	<i>L. cinereus</i>	U68-01	WILD	Indian Canyon, Duchesne Co., Utah	39.9846	-110.6069
03,10	43	CIN_UT012	<i>L. cinereus</i>	U74-01	WILD	Dugway 3, Tooele Co., Utah	40.1873	-112.8091
03,03	43	CIN_UT013	<i>L. cinereus</i>	U28-02	WILD	Island Park Jct, Uintah Co., Utah	40.4926	-109.3853
03,10	40	CIN_UT014	<i>L. cinereus</i>	U69-02	WILD	Echo Canyon, Summit Co., Utah	41.0457	-111.3117
03,03	41	CIN_UT015	<i>L. cinereus</i>	U66-02	WILD	Rabbit Springs, Boxelder Co., Utah	41.3963	-113.8593
03,03	43	CIN_UT016	<i>L. cinereus</i>	Acc-379	WILD	Garland, Utah	41.7500	-112.3333
03,03	43	CIN_UT017	<i>L. cinereus</i>	Acc-374	INC	Benson, Utah	41.8333	-111.8333
03,25	43	CIN_UT018	<i>L. cinereus</i>	Acc-375	INC	Benson, Utah	41.8333	-111.8333
03,26	43	CIN_UT019	<i>L. cinereus</i>	Acc-376	INC	Benson, Utah	41.8333	-111.8333
03,03	46	CIN_UT022	<i>L. cinereus</i>	U71-02w	WILD	Pocatello Valley W. Boxelder Co., Utah	41.9662	-112.4241
03,03	40	CIN_UT023	<i>L. cinereus</i>	U77-01	WILD	Bear Lake, Rich Co., Utah	41.9906	-111.2817
03,27	42	CIN_WA001	<i>L. cinereus</i>	T-1004	INC	Whitman NIAS, Wash.	46.0382	-118.4582
03,27	41	CIN_WA003	<i>L. cinereus</i>	T-1000	INC	S Toppenish, Wash.	46.0847	-120.5515
03,27	41	CIN_WA004	<i>L. cinereus</i>	T-996	INC	S Toppenish, Wash.	46.2352	-120.4177
03,34	26	CIN_WA005	<i>L. cinereus</i>	T-1003	INC	Prescott, Wash.	46.2917	-118.2722
03,20	48	CIN_WA006	<i>L. cinereus</i>	T-998	INC	S Mesa, Wash.	46.4750	-119.0082
29,01	27	CIN_WA007	<i>L. cinereus</i>	T-996	INC	E Starbuck, Wash.	46.5053	-117.8892
03,27	41	CIN_WA008	<i>L. cinereus</i>	T-995	INC	Dodge, Wash.	46.5230	-117.8340
25,35	41	CIN_WA009	<i>L. cinereus</i>	T-14	WILD	Wawawai Park, Wash.	46.6333	-117.3633
03,01	41	CIN_WA010	<i>L. cinereus</i>	T-997	INC	Washluona / Kahlofus, Wash.	46.6902	-118.4223
56,03	42	CIN_WA011	<i>L. cinereus</i>	T-992	INC	E Washluona, Wash.	46.7000	-118.3687
03,01	41	CIN_WA012	<i>L. cinereus</i>	T-991	INC	Washluona, Wash.	46.7597	-118.3377
03,32	41	CIN_WA013	<i>L. cinereus</i>	T-993	INC	Hopper / Lacrosse, Wash.	46.7672	-118.0258
56,03	28	CIN_WA014	<i>L. cinereus</i>	T-994	INC	Dusty, Wash.	46.7899	-117.7037
03,01	42	CIN_WA015	<i>L. cinereus</i>	T-1014	INC	SW Lacrosse, Wash.	46.7833	-117.9500
63,64	41	CIN_WA016	<i>L. cinereus</i>	T-971	INC	Colfax, Wash.	46.9032	-117.3513
03,01	41	CIN_WA017	<i>L. cinereus</i>	T-1012	INC	Lind / Ralston, Wash.	46.9167	-118.4500
03,27	48	CIN_WA018	<i>L. cinereus</i>	T-1013	INC	E Lind, Wash.	46.9500	-118.8333
27,24	41	CIN_WA019	<i>L. cinereus</i>	T-972	INC	Steptoe / St. John, Wash.	47.0223	-117.4903
03,27	41	CIN_WA020	<i>L. cinereus</i>	T-973	INC	St. John, Wash.	47.1088	-117.6402
34,65	40	CIN_WA021	<i>L. cinereus</i>	T-974	INC	Lamont, Wash.	47.1865	-117.8895
03,01	41	CIN_WA022	<i>L. cinereus</i>	T-990	INC	Ephrata, Wash.	47.2727	-118.5747
03,01	41	CIN_WA023	<i>L. cinereus</i>	T-982	INC	Lamona, Wash.	47.3167	-118.4333
03,26	41	CIN_WA024	<i>L. cinereus</i>	T-975	INC	Sprague, Wash.	47.3340	-118.0008
03,27	42	CIN_WA025	<i>L. cinereus</i>	T-983	INC	Marlin, Wash.	47.3858	-119.0408
03,03	41	CIN_WA026	<i>L. cinereus</i>	T-976	INC	NW Sprague, Wash.	47.4008	-118.1563
34,65	42	CIN_WA027	<i>L. cinereus</i>	T-984	INC	Stratford, Wash.	47.4215	-119.2902
03,01	45	CIN_WA028	<i>L. cinereus</i>	T-985	INC	Lenore Lake, Wash.	47.4870	-119.5078
35,32	41	CIN_WA029	<i>L. cinereus</i>	T-989	INC	Wilbur / Odessa, Wash.	47.5238	-118.7765
03,27	42	CIN_WA030	<i>L. cinereus</i>	T-977	INC	NE Harrington, Wash.	47.5503	-118.1835
03,27	40	CIN_WA031	<i>L. cinereus</i>	T-986	INC	Soap Lake, Wash.	47.6018	-119.3730
03,01	40	CIN_WA032	<i>L. cinereus</i>	T-980	INC	Spokane, Wash.	47.6390	-117.5696
03,01	41	CIN_WA033	<i>L. cinereus</i>	T-981	INC	Reardan, Wash.	47.6500	-117.8333
03,01	40	CIN_WA035	<i>L. cinereus</i>	T-978	INC	N Davenport, Wash.	47.7755	-118.1707
03,27	41	CIN_WA036	<i>L. cinereus</i>	T-987	INC	S Electric City, Wash.	47.8417	-118.1087
11,27	41	CIN_WA037	<i>L. cinereus</i>	T-1048	WILD	N Coulee Dam, Wash.	48.0377	-118.9678
03,03	42	CIN_WA038	<i>L. cinereus</i>	T-1064	WILD	Monse, Wash.	48.1333	-119.6692
03,27	42	CIN_WA039	<i>L. cinereus</i>	T-1049	WILD	Omak Lake, Wash.	48.2337	-119.3010
03,01	42	CIN_WA040	<i>L. cinereus</i>	T-1082	WILD	W Okanogan, Wash.	48.3337	-119.6635
55,33	41	CIN_WA041	<i>L. cinereus</i>	T-1083	WILD	SE Twisp, Wash.	48.3672	-120.0240
03,01	42	CIN_WA043	<i>L. cinereus</i>	T-1078	WILD	NE Riverside, Wash.	48.5542	-119.4190

Table 2-2. Chloroplast DNA and ITS Haplotype(s), and locality information for 319 *Leymus* accessions. (continued)

haplotypes									
ITS	cpDNA	Sample ID	Species	Accession	Origin	Location	Latitude	Longitude	
03.01	42	CIN_WA044	<i>L. chinensis</i>	T-1077	WILD	N Riverside, Wash.	48.8948	-119.4858	
03.27	41	CIN_WA046	<i>L. chinensis</i>	T-678	INC	Kettle Falls, Wash.	48.5827	-119.0078	
03.01	42	CIN_WA047	<i>L. chinensis</i>	T-1080	WILD	Fish Lake, Wash.	48.0082	-119.5893	
03.25	41	CIN_WA048	<i>L. chinensis</i>	T-1075	WILD	SE Tonasket, Wash.	48.8160	-119.3718	
03.01	40	CIN_WA049	<i>L. chinensis</i>	T-1079	WILD	Tonasket / Riverside, Wash.	48.8355	-119.6380	
03.27	42	CIN_WA050	<i>L. chinensis</i>	T-1074	WILD	Loomis, Wash.	48.8543	-119.6383	
03.03	40	CIN_WY051	<i>L. chinensis</i>	9076036	WILD	LooseSVNC-93-comm, Sweetwater Co., Wash.	44.0297	-110.0289	
03.20	43	CIN_WY052	<i>L. chinensis</i>	Acc:380	WILD	Kemmerer, Wash.	41.8188	-110.5314	
03.03	43	CIN_WY053	<i>L. chinensis</i>	Acc:357, PI 563854	INC	Kemmerer, Wyo.	41.8857	-110.4633	
03.03	42	MAGNAR_3A	<i>L. chinensis</i>	Acc:69	INC	Magnar			
03.03	42	MAGNAR_3C	<i>L. chinensis</i>	Acc:69	INC	Magnar			
03.03	42	MAGNAR_5A	<i>L. chinensis</i>	Magnar-27101	INC				
03.03	56	MAGNAR_5C	<i>L. chinensis</i>	Magnar-27165	INC				
03.03	43	TRAILHEAD_9C	<i>L. chinensis</i>	Trailhead4y27165	INC				
06.21	64	WASHOE_4A	<i>L. chinensis</i>	Washoe	INC	Deer Lodge Co., Mont.	48.1008	-112.9336	
03.21	44	CIN_NV008	<i>L. chinensis</i> (x <i>trifloroides</i> ?)	T-49	INC	Eureka Co., Neva.	39.4957	-115.9509	
20.59	46	CIN_NV020	<i>L. chinensis</i> (x <i>trifloroides</i> ?)	T-58	INC	Secret Pass, Elko Co., Neva.	40.8900	-115.2833	
25.20	41	CIN_NV062	<i>L. chinensis</i> (x <i>trifloroides</i> ?)	D2659					
03.03	45	CIN_OR028	<i>L. chinensis</i> (x <i>trifloroides</i> ?)	T-1657	WILD	E Burns Jm., Oreg.	42.7776	-117.8484	
03.03	41	CIN_OR010	<i>L. chinensis</i> (x <i>trifloroides</i> ?)	T-1656	WILD	5.8 mi NE of Burns Jm., Oreg.	42.7868	-117.7474	
03.03	30	CIN_OR010C	<i>L. chinensis</i> (x <i>trifloroides</i> ?)	T-1655	WILD	5.8 mi NE of Burns Jm., Oreg.	42.7868	-117.7474	
03.03	23	CIN_OR033	<i>L. chinensis</i> (x <i>trifloroides</i> ?)	T-1670	WILD	Mathur Co., Oreg.	44.4200	-118.1140	
03.34	49	CIN_OR034	<i>L. chinensis</i> (x <i>trifloroides</i> ?)	T-1671	WILD	E John Day, Oreg.	44.4308	-118.8458	
62.62	44	TRI_NV007	<i>L. chinensis</i> (x <i>trifloroides</i> ?)	T-48	INC	Eureka Co., Neva.	39.4667	-115.9500	
03.03	43	PAR_4M	<i>L. chinensis</i> x <i>L. trifloroides</i>	TC2					
03.03	44	PAR_6M	<i>L. chinensis</i> x <i>L. trifloroides</i>	TC1					
03.13	41	CONDENSATUS_CA1	<i>L. condensatus</i>	Atkins, CA		7-Sep-93			
03.13	41	CONDENSATUS_CA2	<i>L. condensatus</i>	PI531804		from various locations			
36.52	31	FLAVESCENS_ID1	<i>L. flavescens</i>	KJ38	WILD	Power Co., Idaho	42.7628	-112.9128	
73.74	31	FLAVESCENS_UC1	<i>L. flavescens</i>	R-24-48.6	WILD	R-24-4 & 6: Haseil & Harris			
79.53	32	INNOVATUS_ECA1A	<i>L. innovatus</i>	PI531805			56.0000	-117.0000	
48.54	32	INNOVATUS_UC2A	<i>L. innovatus</i>	PI236820					
03.03	57	MOJ_CA002A	<i>L. mcgavensis</i>	D-3820		CA			
76.77	58	MOLLIS_AK1B	<i>L. mollis</i>	R-21-31-35		R-21-31-35: Alaska; Last seed			
79.80	59	MOLLIS_EU1A	<i>L. mollis</i>	D-4088		Russia: Original seed			
81.51	54	MULTICAULIS_EU1A	<i>L. multicaulis</i>	PI-495521		China			
50.81	54	MULTICAULIS_UC1A	<i>L. multicaulis</i>	D-3773					
50.81	51	SHOSHONE_1A	<i>L. multicaulis</i>	Shoshone'	INC	Brigder PMC, Riverton, Wyo.	43.0101	-108.3782	
48.49	51	SHOSHONE_1C	<i>L. multicaulis</i>	Shoshone'	INC	Brigder PMC, Riverton, Wyo.	43.0101	-108.3782	
45.47	50	RACEMOSUS_EU1	<i>L. racemosus</i>	DJ5801		Russia, Original seed			
45.47	50	RACEMOSUS_UC2	<i>L. racemosus</i>	D-2940		2n=26			
45.45	52	RAMOSUS_EU1A	<i>L. ramosus</i>	PI531812		2n=26, Wash.	48.7482	-120.7867	
45.45	52	RAMOSUS_EU2A	<i>L. ramosus</i>	D-1775		PI448332			
45.45	61	SABULOSUS_EU1A	<i>L. sabulosus</i>	PI499553		Xi njiang			
03.75	82	SAL_UT001	<i>L. salinus</i>	D-3488					
14.23	33	SAL_UT004	<i>L. salinus</i>	SaviersSinks	WILD	Saviers Sinks via Snow, Utah	38.5486	-112.3946	
14.23	33	SAL_UT005	<i>L. salinus</i>	GBRC: US5-01	WILD	Ephraim, Utah	38.7550	-111.3814	
03.23	83	SAL_UT006	<i>L. salinus</i>	PI531817	WILD	Joe's Valley Reservoir	39.3262	-111.2747	
03.23	35	SAL_WY001	<i>L. salinus</i>	KJ-41	WILD		40.3842	-111.3826	
04.22	35	SAL_WY002	<i>L. salinus</i>	PI531815	INC	Synthetic of 8 reps	41.5605	-109.5278	
37.58	38	SECALINUS_EU1	<i>L. secalinus</i>	D-3448	WILD	Green River, WY	41.5605	-109.5278	
45.45	53	SECALINUS_EU2	<i>L. secalinus</i>	JA125		USSR: Seed like racemosus			
45.45	52	SECALINUS_EU3	<i>L. secalinus</i>	JA-129		USSR: Seed looks like racemosus			
37.37	38	SECALINUS_EU4	<i>L. secalinus</i>	PI504485		PMC			
37.37	38	SECALINUS_EU5	<i>L. secalinus</i>	PI531819		2n=26: PRC			
03.58	44	PAR_1T	<i>L. trifloroides</i>	LTFO3	WILD	NV, ID border: Original, 1987	42.8750	-115.9462	
03.57	44	PAR_OR001A	<i>L. trifloroides</i>	Acc:641	WILD	Jamieson, Oreg.	44.1867	-117.4687	
70.71	46	RIO_4A	<i>L. trifloroides</i>	RIO-490369	INC	Rio, Calif.	38.1827	-121.1803	
70.71	46	RIO_4C	<i>L. trifloroides</i>	RIO-490369	INC	Rio, Calif.	38.1827	-121.1803	
03.57	44	TRI_ID002	<i>L. trifloroides</i>	KJ25	WILD	SW of Brunsell, Owyhee Co., Idaho	42.876	-115.8482	
52.72	41	TRI_ID002C	<i>L. trifloroides</i>	KJ25	WILD	Neva-Idaho border	42.876	-115.8482	
20.59	44	TRI_ID003	<i>L. trifloroides</i>	Acc:842	WILD	Malba, Owyhee Co., Idaho	43.3754	-116.5290	
62.72	44	TRI_NV001	<i>L. trifloroides</i>	Acc:840		Oravada, Humboldt Co., Neva.	40.6167	-119.9000	
62.72	44	TRI_NV002	<i>L. trifloroides</i>	PI531822	WILD	Indian Colony Co., Neva.	40.6167	-116.9000	
03.03	47	TRI_NV003C	<i>L. trifloroides</i>	T-1668	WILD	Quinn River Crossing, Humboldt Co., Neva.	41.5920	-116.4317	
57.80	44	TRI_NV004	<i>L. trifloroides</i>	KJ22	WILD	Neva.	42.1595	-110.1159	
03.01	44	TRI_NV005	<i>L. trifloroides</i>	D2663		Neva.			
03.62	41	TRI_NV006	<i>L. trifloroides</i>	T22152acc		Neva.			
03.57	44	TRI_OR001A	<i>L. trifloroides</i>	T-1695	WILD	Oreg.	42.8793	-116.9880	
62.72	41	TRI_OR003	<i>L. trifloroides</i>	T-1694	WILD	Rome, Malheur Co., Oreg.	42.8400	-117.6250	
52.72	41	TRI_OR003C	<i>L. trifloroides</i>	T-1694	WILD	Rome, Malheur Co., Oreg.	42.8400	-117.6250	
03.57	41	TRI_OR004	<i>L. trifloroides</i>	T-1693	WILD	Malheur National Wildlife Refuge, Malheur Co., Oreg.	43.1378	-116.9130	
62.72	44	TRI_OR005	<i>L. trifloroides</i>	T-1698	WILD	Malheur River Canyon, Malheur Co., Oreg.	43.7778	-117.7883	
62.72	41	TRI_OR005C	<i>L. trifloroides</i>	T-1696	WILD	Malheur River Canyon, Malheur Co., Oreg.	43.7778	-117.7883	
62.62	44	TRI_OR007	<i>L. trifloroides</i>	T-1698	WILD	Jamieson, Oreg.	44.1910	-117.4515	
57.69	37	TRI_OR008C	<i>L. trifloroides</i>	T-1597	WILD		42.3287	-116.7864	
66.21	43	WASHOE_5A	<i>L. chinensis</i>	Washoe	INC	Deer Lodge Co., Mont.	48.1008	-112.9336	
-	-	AJ608151	<i>Psathyrostachys juncea</i>	AJ608151		Genbank			
-	-	Bozlosky	<i>Psathyrostachys juncea</i>		INC	Russia: Kevin Jensen			
-	-	Mankota	<i>Psathyrostachys juncea</i>		INC	Russia: Kevin Jensen			
-	-	L36485.1.67	<i>Thinopyrum elongatum</i>	L36485.1.67		Genbank			
-	-		<i>Thinopyrum elongatum</i>						
-	-		<i>Thinopyrum ponticum</i>						

DNA extractions- A minimum of four plants from each accession were germinated on blotter paper and grown in single-plant containers in the USDA-ARS, Forage and Range Research Laboratory greenhouse at Utah State University, Logan, UT. DNA was obtained using the DNAeasy plant DNA isolation kits (Qiagen Inc., Valencia, California) from fresh tissue of two individual plants per accession. More intensive sampling was performed on several groups of accessions to identify hybridization between species. Referred to as “paired sites,” these regions were identified as localities where *L. cinereus* and *L. triticoides* grow collectively.

Evaluation of candidate cpDNA primers- The informative value of four chloroplast DNA primers (*trnH-psbA*, *rpl36-rps8*, *trnK-rps16*, *trnC-ycf6* (*petN*)) were tested for amplification efficiency, amplicon length, and sequence divergence rates for three species of *Leymus* following the same procedure described under the ‘sequencing’ methods heading (see below). The *trnH-psbA* and *trnK-rps16* primers were selected based on the potential displayed in this pilot study and the findings of other surveys in search of non-coding plastid DNA (Kress et al., 2005; Shaw et al., 2005). It was noted that the limited size (574-632 bp) of sequence reactions may have been too small to document distinct genetic differences within accessions or populations (Table 2-4). Still, the expectation was that the combination of cpDNA and ITS region analysis data would be sufficient to detect specific polymorphic differences between species, providing enough informative polymorphic sites to construct a *Leymus* phylogeny.

Sequencing- Sequencing was performed for cpDNA and ITS PCR products. The Quickstep 2 PCR and the ExcelaPure 96-well UF PCR purification kits (Edge-Biosystems, Gaithersburg, Maryland) were used to purify PCR products prior to

sequencing (see Appendix B). Chloroplast PCR product size ranged from 600 to 650 bp, requiring 32 ng of DNA amplification product and 1 μ l of 2 pmol/ μ L primer (2 μ M) for each sequencing reaction. The -4, -L (White et al., 1990) ITS region was approximately 600 bp. Approximately 40 ng of the ITS amplification product and 1 μ l of 2 pmol/ μ L primer (2 μ M) for each sequencing reaction. Sequencing reactions were carried out 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, Gaithersburg, Maryland). The retained elutes from this final purification were loaded onto the ABI3730 for sequence analysis.

Sequence alignment and indel coding- The overlapping sequences from complementary strands for each sample were aligned and manually inspected in SEQUENCHER 4.5 and 4.6 (Gene Codes Corporation, Ann Arbor, Michigan). Consensus sequences from complementary strands of each plant were submitted to Genbank. The chloroplast genome is inherited as a single unit without recombination, thus sequences from the *trnH-psbA* and *trnK-rps16* coding regions were concatenated into a single sequence for each sample (Soltis et al., 1996; McKenzie et al., 2006). The large size of the data set required that the phylogeny be expressed in terms of haplotypes rather than individual accessions for both the cpDNA and ITS phylogenies. Haplotypes were detected by contiging identical sequences. Alignment of the haplotypes was conducted using MEGA version 3.1 (Kumar et al., 2004). Binary numbers were used to code for indels using a simple method described by Simmons and Ochoterena (2000). Sequences were collapsed into haplotypes prior to the exclusion of complex indel regions

in further analyses. Information for each accession including haplotype, species, and locality for cpDNA and ITS can be found in Table 2-2.

The original ITS sequences included many sites with mixed-base signals (heterozygous polymorphisms) and relatively few fixed homozygous polymorphisms. *Leymus* is an outcrossing polyploid, which may explain the high levels of heterozygosity found in the nuclear genome. These alleles were separated so that allele-specific-characterization could be assessed (Zhang and Hewitt, 2003). Heterozygous sites were reduced into haplotypes with the statistical Bayesian method PHASE 2.1, introduced by Stephens et al. (2001) and Stephens and Scheet (2003). This program uses Markov chain Monte Carlo (MCMC) to reconstruct haplotypes from population genotype data. Prior information (the assumed patterns of haplotypes) is combined with the likelihood (based on the observed data) to determine the posterior distribution (the conditional distribution of unobserved haplotypes given the observed haplotypes data). The most likely pairs of haplotypes were determined for each individual.

Phylogenetic analysis- Chloroplast and ITS haplotypes were analyzed separately in PAUP version 4.0b10 (Swofford, 2000) with parsimony and distance analysis. Phylograms were constructed using a heuristic search method with 1000 random replicates, holding one tree at each step with tree-bisection-reconnection (TBR) swapping. Bootstrap values were replicated 1000 times. Neighbor-joining (NJ) (Saitou and Nei, 1987) and the Un-weighted Pair Grouping Method (UPGMA) (data not shown) were used with 1000 bootstrap replicates to evaluate genetic distance between haplotypes. The efficiency of the barcoding genes in detecting polymorphisms that

distinguish operational taxonomic units (OTU's) or species was tested with comparisons of pairs of (OTU's) using analysis of molecular variance (AMOVA) statistics; e.g. F_{st} statistics (F_{st}), significance testing, average pairwise comparisons, and haplotype frequency estimations. Total distance and Kimura's (1980) two-parameter distances (K2P) were used as a measure of differences between haplotypes. The K2P test outputs a corrected percentage of nucleotides for which two haplotypes are different, allowing for multiple substitutions per site. This takes into account substitution rates between transitions and transversions (Kimura, 1980). Phylograms were constructed based on the average pairwise differences between species ($PiXY$), average number of pairwise differences within species (PiX and PiY), corrected average pairwise differences among species ($PiXY - (PiX + PiY) / 2$), and the corresponding apportionment of variation (AMOVA) based on Euclidean distances computed using Arlequin (Excoffier et al., 1992).

Divergence time estimates using calibrated molecular clock- Kimura's 2-parameter average pairwise differences were calculated for the 85 ITS haplotypes and 64 cpDNA haplotypes and analyzed in AMOVA to determine F_{st} and the number of nucleotide substitutions per site. Kimura two-parameter distance measures were converted into a simple molecular clock using $K=2Tk$, T is the divergence time, k is the constant rate of nucleotide substitution (Kimura, 1981), and K is the corrected total number of base substitutions per site that separate two sequences. Divergence estimates of about 10 million years ago (My) for wheat and maize (Stebbins, 1981) have been used to calibrate divergence times among various grass lineages (Ogihara et al., 1991; Charmet et al., 1997; Fjellheim et al., 2006). Estimates of cpDNA divergence time were calculated

for North American and Eurasian taxa based on K2P corrected distances for the two most closely related species between the two groups, *L. innovatus* of North America and *L. chinensis* from Asia. Divergence time estimates were based on the constant rates of substitution (k) for Triticeae grasses used by Ogihara et al. (1991), where the substitution rates ranged from 3.75×10^{-9} for *Triticum aestivum* and *Oryza*, to 1.33×10^{-8} for *Aegilops crassa* and *Triticum aestivum*.

Genetic and geographic distance correlation- Pairwise comparisons of the total number of differences between 247 *L. cinereus* accessions were computed using PAUP 4.0b10. Geographic coordinates for each accession were converted to geographic distances (km) between accessions with SAS software (SAS Institute Inc. Cary, North Carolina) using the formula: $\text{km} = r \cdot \arccos [\cos(\text{LATX}) \cos(\text{LONGX}) \cos(\text{LATy}) \cos(\text{LONGy}) + \cos(\text{LATX}) \sin(\text{LONGX}) \cos(\text{LATy}) \sin(\text{LONGy}) + \sin(\text{LATX}) \sin(\text{LATy})]$, where LATX, LONGX and LATy, LONGy are the latitude and longitude (expressed in radians) for the two accessions (X and Y) and r is 6378 km, the radius of Earth.

Genetic and geographic distance was correlated using the Mantel (1967) test statistic (Z), using the Mxcomp procedure of NTSYS-pc (Rohlf, 1998). Significance tests for these correlations were determined by comparing observed values to values obtained by 1000 random permutations (Smouse et al., 1986). Therefore, the upper-tail probability (p) that 1000 random Mantel test-statistic (Z) values are (by chance) less than observed values of Z equals 0.002 or greater.

RESULTS

Information value of candidate cpDNA primers- Of the four chloroplast DNA primers tested, the *trnH-psbA* and *trnK-rps16* primers showed the highest divergence among species and most informative value (600 to 1000 base pairs) (Table 2-3) (Kress et al., 2005). Poor sequence length reads warranted removal of the *trnC-ycf6s* primers from consideration. The *trnK-rps16* (655 bp) and *trnH-psbA* (630 bp) primer pairs displayed the greatest divergence values among taxa. These two primers were selected based on the potential displayed in this pilot study and the findings of other surveys in search of non-coding plastid DNA for barcoding purposes (Kress et al., 2005; Cowan et al., 2006).

cpDNA sequences- Characteristics of all sequences are summarized in Table 2-4. Ninety-eight problematic indels were removed from the combined cpDNA sequence analysis. The *trnH-psbA* region sequences were approximately 574-608 bp in length before alignment and 661 bp in length after alignment in Mega 3.1. For the analysis 32 bp characters were eliminated, including 26 bp ambiguous indels, and a 6 bp palindromic repeat, that occurred in nearly half of the samples. Palindromic repeats are two regions

Table 2-3. Pilot evaluation of sequence divergence among four intergenic spacers, % sequence divergence is based on the total number of indels and nucleotide substitutions for the aligned length.

Primers	length (bp)	% sequence divergence	variable sites
<i>trnH-psbA</i>	620	0.655	4
<i>rpl36-rps8</i>	540	0.5	3
<i>trnK-rps16</i>	620	1.29	8
<i>trnC-petN</i> (ycf6)	900	-	-

Table 2-4. Sequence characteristics of cpDNA and ITS sequences.

Sequence Characteristic	<i>psbA-trnH</i>	<i>trnK-rps16</i>	combined cpDNA sequences	ITS no phase haplotypes	ITS phase haplotypes
Length range (nucleotides)	574-632	581-622	1155-1254	596-599	596-599
Aligned length	661	693	1354	610	610
character eliminated	32	66	98	15	15
indels coded	9	10	19	4	4
final sequence length	638	637	1275	595	595
# parsimony informative sites	13	41	54	25	47
# parsimony uninformative sites	13	21	34	5	31
TOTAL # of variable sites	26	62	88	30	78
Tree Length	32	79	114	35	150
Consistency Index	0.8571	0.8485	0.807	1	0.5933
Retention Index	0.9775	0.9817	0.9638	1	0.779

of a nucleic acid molecule, which have the same nucleotide sequence but in an inverted orientation from a central point of symmetry. They contain exactly the same coding message read in either direction (www.biochem.northwestern.edu/holmgren/Glossary/Definitions). The palindromic repeats were removed from the analysis to reduce the level of ambiguity in parsimony and distance analysis. The final sequence length for the *trnH-psbA* primer had 26 variable sites, 13 parsimony uninformative, and 13 parsimony informative sites, nine of which were indels. The *trnK-rps16* cpDNA sequences were approximately 581-587 in length and 693 after alignment. Sixty-seven ambiguous characters were removed from the analysis. The final sequence length was 627 bp, with 21 bp parsimony-uninformative, and 41 bp parsimony informative sites. Ten of the informative characters were indels. When combined, the two chloroplast primers had 88 potentially informative characters, of which 54 bp were parsimony-informative and 34 bp parsimony-uninformative, with a total aligned sequence length of 1275 bp for 334 total sequences, including four outgroup reference specimens (two *Psathyrostachys juncea* varieties, *Thinopyrum elongatum* and *Thinopyrum ponticum*). Sixty-four haplotypes were detected among the *Leymus* cpDNA (Appendix C) sequences.

cpDNA sequence AMOVA statistics- Genetic variation within and among species was tested with AMOVA (F_{st} and average pairwise comparisons of species pairs) using the total number of differences (Appendix D) and the K2P-corrected number of pairwise differences between haplotypes (Appendix E). The K2P pairwise sequence divergence among *Leymus* haplotypes ranges from 0 to 1.9 %. When analyzed as groups, 84.53% of the total variation was attributed to differences between North American and Eurasian taxa. 7.46% variation was attributed to differences among taxa within groups and 8.01% variation was attributed to differences within taxa.

Of nine North American species sampled, three taxa; *L. flavescens* (haplotype '31'), *L. innovatus* (haplotype '32'), and Alaskan *L. mollis* (haplotype '76' and '77') exhibited species-specific polymorphisms. Still, the majority of comparisons (based on pairwise differences) between these taxa and other *Leymus* were non-significant. Conversely, five species that did not demonstrate species-specific polymorphisms including, *L. ambiguus*, *L. cinereus*, *L. cinereus* x *L. triticoides*, *L. salinus*, and *L. triticoides*, had a higher proportion of significant differences when compared with other species. A significant difference ($F_{st} = 0.1528$) ($p \leq 0.05$) in total average pairwise differences between *L. cinereus* and *L. triticoides* ($PiXY = 2.1952$) was slightly lower than the average number of differences within *L. cinereus* ($PiX = 2.2482$) and higher than the number of differences within *L. triticoides* ($PiX = 1.31429$). Twenty-seven of 36 haplotypes characterizing *L. cinereus*, were unique to *L. cinereus* alone. Of the 27 unique haplotypes, 20 were comprised of a single *L. cinereus* sequence. Haplotype '40' characterized 43 *L. cinereus* accessions, with the greatest frequency (0.1654) among haplotypes unique to *L. cinereus*. Haplotype '40' is different from the highest frequency

haplotypes '41' and '44' (among *L. cinereus*, *L. triticoides*; and putative hybrid sequences) by two, A to C transition mutations. Haplotype '41' characterizes 80 *L. cinereus* sequences (freq. = 0.3077) and six *L. triticoides* sequences (freq. = 0.2857). Haplotype '44' characterizes 11 *L. triticoides* sequences (freq. 0.5238), and 19 *L. cinereus* sequences (freq. = 0.0808). Haplotype '46' characterized two Rio (*L. triticoides*) sequences, two *L. cinereus* sequences, and one putative hybrid, which varied from haplotype '40' by one T to C transversion mutation. Haplotype '46' varied by one T to C transversion in both haplotypes '41' and '44', as well as one A to C transition mutation in haplotype '44' and two A to C transition mutations in haplotype '41'.

The average number of differences within *L. cinereus* x *L. triticoides* hybrids ($PiX = 3.4667$) was actually greater than the number of differences between *L. cinereus* x *L. triticoides* hybrids and *L. cinereus* ($F_{st} = 0.03621$) ($PiXY = 2.8846$). The average number of differences within *L. cinereus* x *L. triticoides* hybrids ($PiX = 3.4667$) was actually greater than the number of differences between *L. cinereus* x *L. triticoides* hybrids and *L. triticoides* ($F_{st} = 0.15283$) ($PiXY = 2.1952$). A higher number of average pairwise differences within the hybrids than between hybrids and other taxa may indicate varying degrees of parental influence from *L. cinereus* and *L. triticoides* among different accessions. All but one *L. cinereus* x *L. triticoides* hybrid haplotype is shared with *L. cinereus*, and six out of eleven haplotypes are shared with *L. triticoides*. Comparisons between *L. cinereus* and *L. cinereus* x *L. triticoides* were non-significant ($p = 0.09$), whereas *L. triticoides* and hybrid comparisons, ($p = 0.03$) were significant.

Although *L. ambiguus* (PiX)=2.8000 has the second highest number of within species average pairwise differences, observations from the cpDNA topologies suggest it

is a well supported North American OTU. The large number of differences can be attributed to haplotype '5' that is separated from the other *L. ambiguus* haplotypes by four to five mutations. Analysis of molecular variance comparisons show significant pairwise F_{st} values between *L. ambiguus* and 12 of 18 other *Leymus* taxa. Non-significant differences between North American *Leymus* and *L. ambiguus* included;

L. innovatus (F_{st} = 0.6250) (PiXY = 5.8333), *L. salinus ssp. mojaveensis* (F_{st} = 0.52000) (PiXY = 5.83333), and *L. salinus* (F_{st} = 0.2041) (PiXY = 5.4444).

L. salinus has the highest number of within pairwise differences (PiX = 5.8667) among *Leymus* tested. *L. salinus* varied by as many as 11 mutational differences between accessions. Non-significance values between *L. salinus* and other *Leymus* taxa included *L. ambiguus* (F_{st} = 0.2041) (PiXY = 5.4444), *L. innovatus* (F_{st} = -0.01538) (PiXY = 4.0000), and *L. salinus ssp. mojaveensis* (F_{st} = 0.9910) (PiXY = 4.0000).

The branch within the North American clade that most closely relates to European and Asian *Leymus* outgroups, is that of two *L. mollis* haplotypes: namely 'haplotype 58', consisting of one Alaskan accession, and 'haplotype 59', consisting of one Russian accession. Significant differences occurred between *L. mollis* and several North American taxa including, *L. ambiguus* (F_{st} = 0.7470) (PiXY = 7.4333), *L. cinereus* (F_{st} = 0.6625) (PiXY = 6.0808), *L. salinus* (F_{st} = 0.3744) (PiXY = 7.0000), and *L. triticoides* (F_{st} = 0.8108) (PiXY = 6.3333).

The lack of significant difference between many *Leymus* species corresponds with taxa for which there were fewer samples e.g. *L. condensatus* (n=2), *L. salinus ssp. mojaveensis* (n=1), *L. mollis* (n=2), *L. flavescens* (n=2), and *L. innovatus* (n=2). The utility

of cpDNA barcodes as a tool for species identification may be dependent on a large enough set of samples in addition to greater than two cpDNA loci.

Patterns in cpDNA phylogenies- Heuristic parsimony and distance neighbor-joining phylogenies yielded similar groupings for the 64 *Leymus* haplotypes and outgroup Triticeae taxa analyzed. The cpDNA heuristic parsimony tree (Figure 2-2), had 114 steps with a consistency index = 0.8070 and a retention index = 0.9638. In order to describe the relationship of North American *Leymus* taxa, haplotypes were subjectively divided into three clades. Several accessions do not group in any specific category. The most extensively sampled taxon, *L. cinereus*, is present in Clades II and III. Clade I is comprised of six *L. ambiguus* haplotypes, two replicates of the same *L. salinus* accession (a suspect hybrid, *L. ambiguus* x *L. salinus*), and one *L. flavescens* haplotype. Clade II is comprised of three Utah *L. salinus* accessions, one California *L. salinus* ssp. *mojavensis* accession, and several *L. cinereus* accessions ranging from British Columbia, Washington, Oregon, and Nevada. Haplotype '29', composed of British Columbia accessions was seven steps from the nearest haplotype. Clade III included *L. cinereus*, *L. condensatus*, *L. salinus*, all *L. triticoides* haplotypes, as well as Magnar, Rio, Washoe, and Trailhead releases, with one to four steps in between haplotypes. All but two putative hybrids (*L. cinereus* x *L. triticoides*) also grouped in Clade III. Clade III OTUs encompass the entire collection area from southern Utah, west to California, east to Montana, and north to British Columbia. *Leymus innovatus* and *L. mollis* do not clade specifically with any of the designated clades. CpDNA heuristic parsimony and neighbor-joining phylogenies show these taxa most closely group with one another (Figure 2-3).

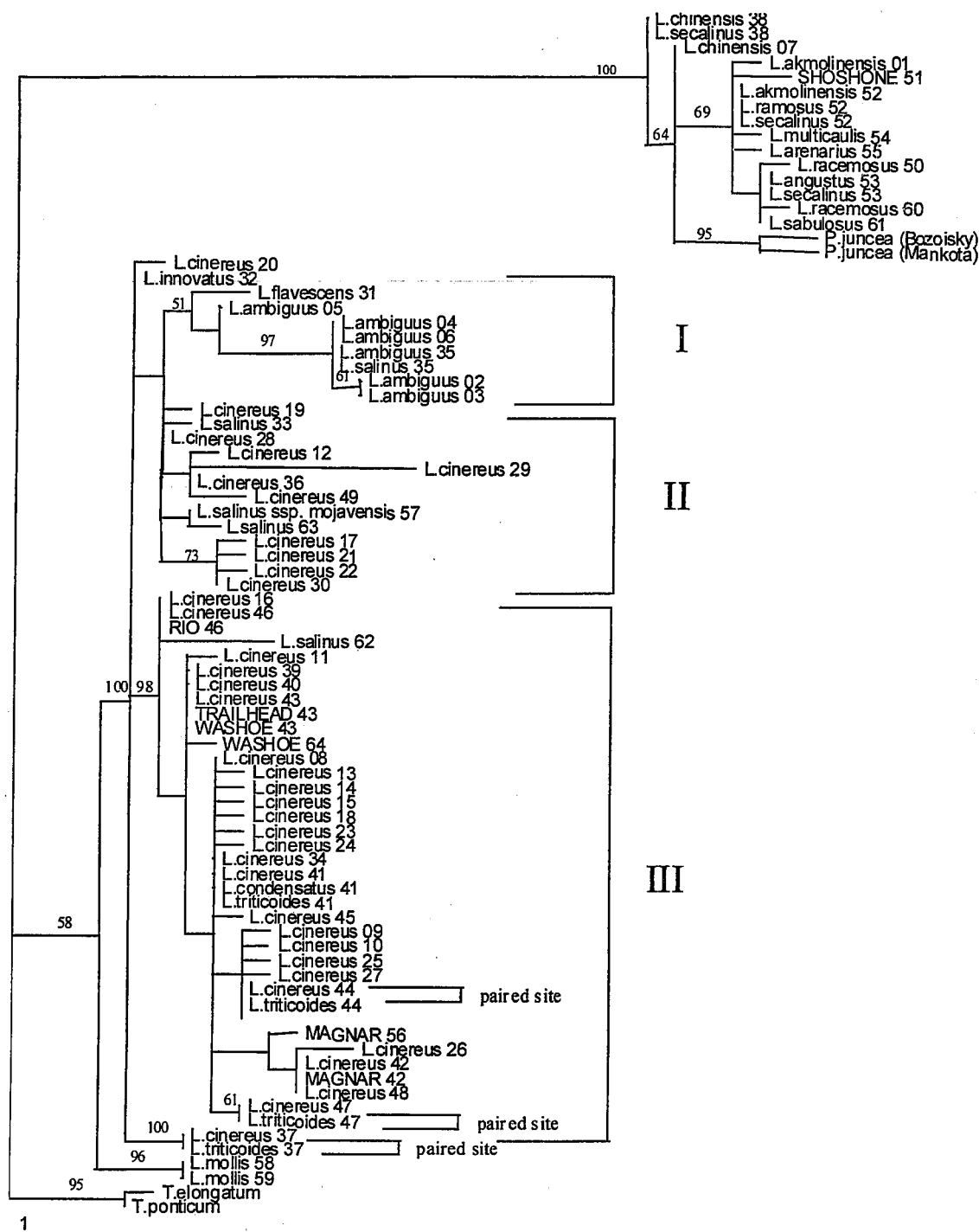


Figure 2-2 Heuristic parsimony analysis for 64 *Leymus* haplotypes and four other Triticeae taxa, based on the chloroplast *trnH-psbA* and *trnK-rps16* spacers, phylogeny is 114 steps with CI= 0.8070 and RI= 0.9638, with bootstrap support values determined from 500 sample replicates.

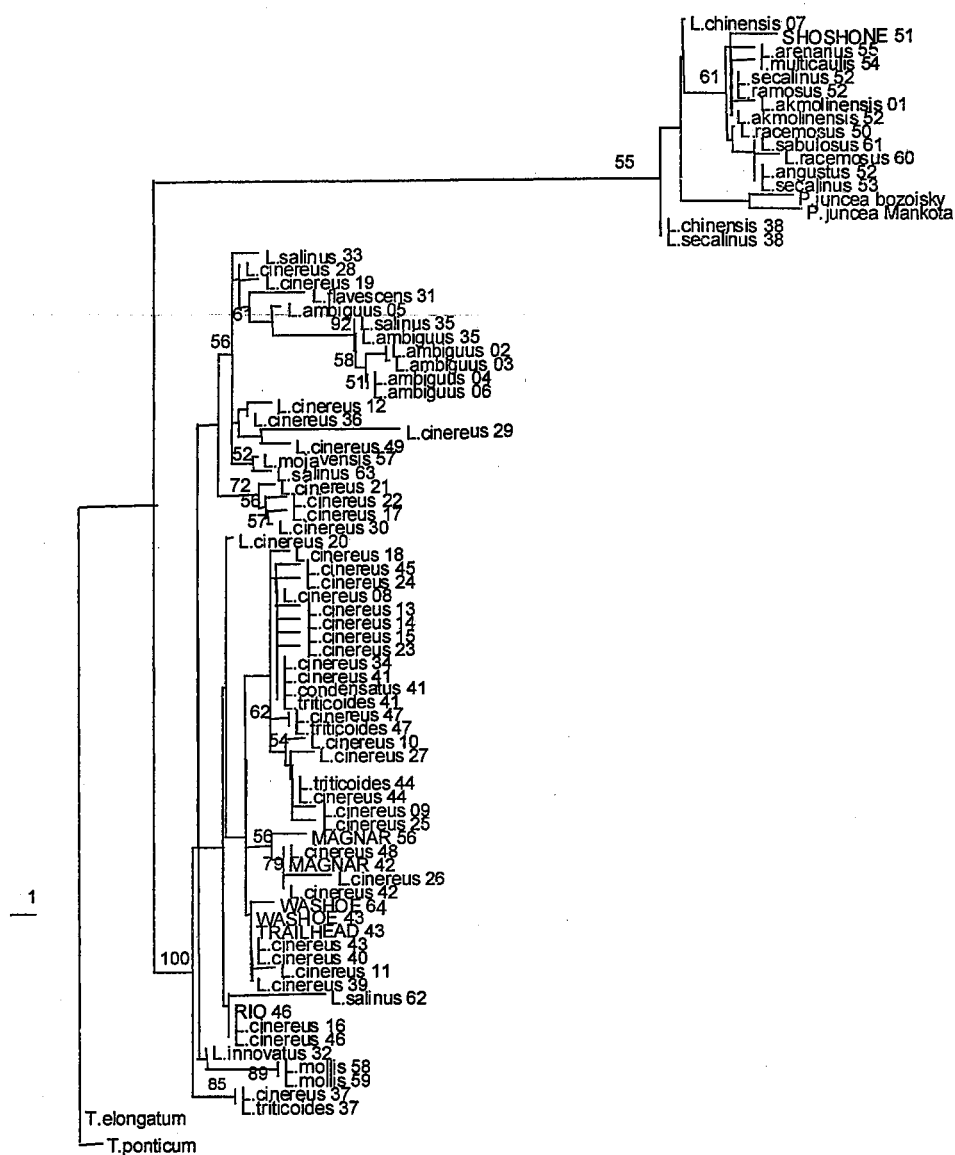


Figure 2-3 Neighbor-joining distance analysis for 64 *Leymus* haplotypes, and four other Triticeae taxa based on the total number of differences (substitutions or indels) among the chloroplast *trnH-psbA* and *trnK-rps16* spacers DNA sequences, with bootstrap support values determined from 1000 sample replicates.

Triticeae outgroup taxa *Thinopyrum elongatum* and *T. ponticum* cluster six to eight steps from the from the closest North American *Leymus* accessions, whereas the two varieties of *Pasthyrostacys juncea*, Bozoisky and Mankota, are 30 steps from the main stem of North American taxa clusters (Appendix D). Phylogenies developed from average pairwise differences using the Kimura 2-parameter test, show only slight differences between North American *Leymus* taxa, less than 1% divergence between taxa (Figure 2-4). *Leymus salinus*, *L. salinus* ssp. *mojavensis*, *L. ambiguus*, and *L. flavescens* cluster, while *L. cinereus*, *L. triticoides*, *L. cinereus* x *L. triticoides*, and *L. condensatus* form another group. *Leymus mollis* and *L. innovatus* are also closely aligned. Interestingly, both *Thinopyrum elongatum* and *T. ponticum* grouped closest to the North American *Leymus* clade, rather than clustering with the “Eurasian” outgroups, as may have been expected.

Simple molecular clock estimation- Divergence time between North American and Eurasian *Leymus* species was estimated based on the K2P-corrected total number of base substitutions that separated the two most recently diverged North American and Eurasian species, *L. innovatus* and *L. chinensis* ($K=0.01718$). Using the same constant rates of nucleotide substitution (k) as Ogiwara et al. (1991), the divergence time between North American and Eurasian taxa was estimated to be between 650 000 years ago ($k = 1.33 \times 10^{-8}$), and 2.3×10^6 mya ($k = 3.75 \times 10^{-9}$).

ITS sequences- Characteristics of all ITS sequences are summarized in Table 2-4. The ITS sequences included 19 *Leymus* taxa and two Genbank Triticeae reference specimens; namely *Psathyrostachys juncea* AJ608151_86 and *Thinopyrum elongatum* L36495.1_87. The ITS region displayed 596-599 bp prior to alignment and 610 bp after

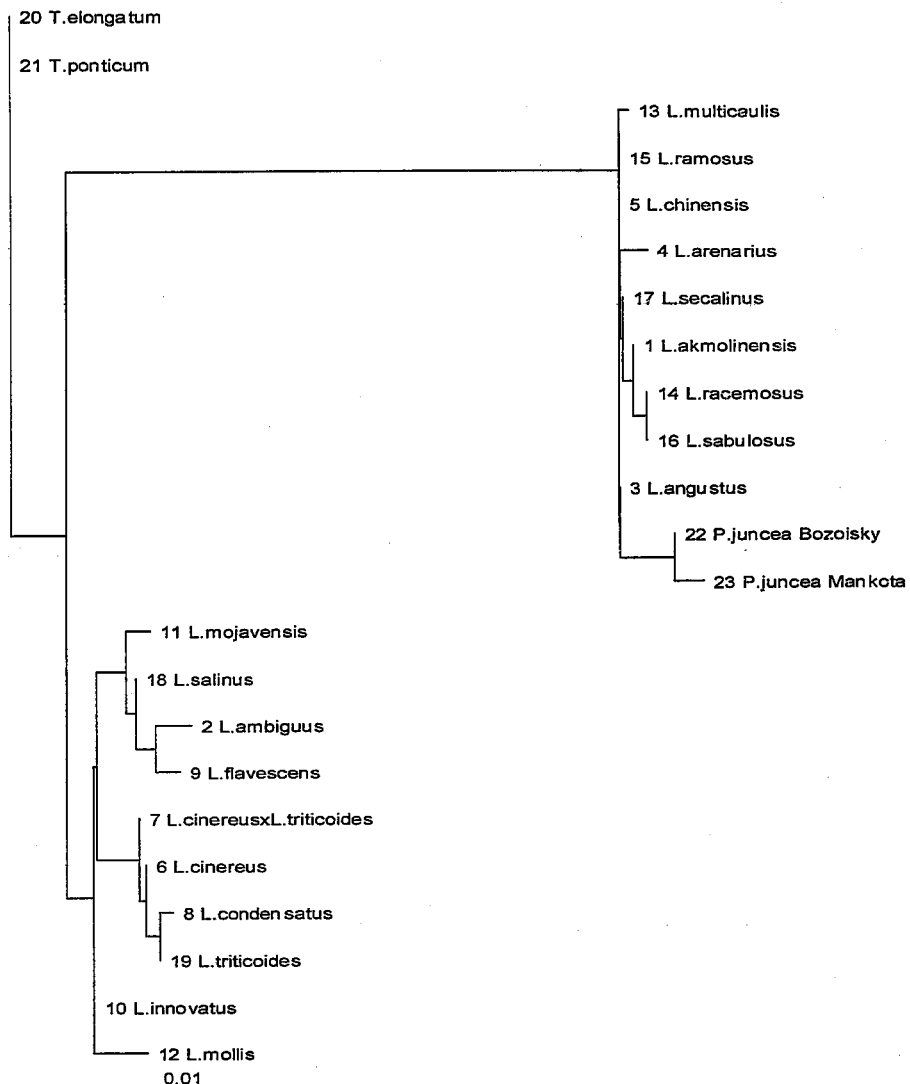


Figure 2-4. Neighbor-joining distance phylogeny based on the cpDNA Kimura two-parameter corrected average number of pairwise differences for 19 *Leymus* taxa.

alignment. Fifteen problematic indels were removed from the ITS sequences. Phased ITS data had 78 potentially informative characters for 604 bp analyzed, of which 47 bp sites were parsimony-informative and 31 bp sites were parsimony-uninformative for 19 *Leymus* taxa and two outgroup taxa, *Thinopyrum elongatum* and *Psathyrostachys juncea*. Comparisons of phased ITS sequences among 10 North American taxa only, had 52 potentially informative characters for 604 bp analyzed, with 29 bp site parsimony-

informative and 23 bp sites parsimony-uninformative. Without the PHASE 2.1 method, ITS sequences for all *Leymus* taxa had only 30 bp potentially informative characters, of which 25 bp sites were parsimony-informative, and 5 bp sites parsimony-uninformative (Table 2-4). Eighty-five haplotypes were detected in the ITS regions for 319 samples using SEQUENCHER 4.6. Two hundred-five of 319 *Leymus* accessions displayed two or more haplotypes. The most common haplotype '03' characterized at least one phase in 240 of 319 accessions among five species of *Leymus*. One hundred four accessions were characterized by haplotype '03' only. Haplotype '03' was separated from 32 other haplotypes by one step, and 23 haplotypes by two steps.

ITS sequences and AMOVA statistics- The efficiency of ITS barcoding sequences was evaluated with AMOVA statistics. The prevalence of non-significant differences based on pairwise comparisons between taxa observed in the cpDNA AMOVA data set, differed from ITS results. A lower percentage of variation (46.78%) was attributed to differences between North American and Eurasian taxa for ITS sequences, and higher percentages were reported for comparisons between taxa within North American and Eurasian groups (31.64%), as well as within individual taxa (21.58%). Overall, there was a higher proportion of significant differences among North American taxa, as well as between *Leymus* outgroups in ITS analyses. Nucleotide sequence divergence based on the K2P corrected average number of pairwise differences among *Leymus* ranged from 0-15.7% (Appendix G). As in the cpDNA sequences, three North American taxa, *L. flavescens*, *L. innovatus*, and *L. mollis*, showed unique polymorphisms. There were two collection sites for each of the three taxa. All three taxa resulted in four different allelic haplotypes. Several of these haplotypes displayed higher

numbers of differences between haplotypes of the same species than when compared to haplotypes comprised of different species. *Leymus innovatus* haplotype '78' has as many as eleven mutations separating it from the three other *L. innovatus* haplotypes. Haplotype '78' has fewer mutations (only 2-4) when compared to the four *L. mollis* haplotypes '76', '77', '79' and '80'. *Leymus flavescens* also has four haplotypes of equal frequency (0.25). The four haplotypes are differentiated by one to five mutation differences. *Leymus flavescens* haplotype '36' has as few as one mutation difference from other various haplotypes among several *Leymus* taxa including *L. ambiguus*, *L. cinereus*, *L. salinus*, and *L. triticoides*.

The average number of ITS differences within *L. cinereus* (PiX = 0.9839), *L. salinus* (PiX = 1.8182), *L. ambiguus* (PiX = 1.4546), and *L. triticoides* (PiX = 1.2834) are lower than that observed in cpDNA. In contrast, several taxa that had zero within pairwise differences in the cpDNA, had higher within-pairwise difference values in phased ITS comparisons, including *L. condensatus* (PiX = 2.0000), *L. flavescens* (PiX = 1.8333), *L. innovatus* (PiX = 5.3333), *L. salinus ssp. mojaveensis* (PiX = 1.0000), and *L. mollis* (PiX = 3.0000).

AMOVA comparisons of pairs of ITS OTUs based on total distance (Appendix F) showed a significant difference ($p \leq 0.05$) for average pairwise differences between *L. cinereus* and *L. triticoides* ($F_{st} = 0.32967$) (PiXY) = 1.4789. The between OTU comparison was higher than the average number of differences within *L. cinereus* (PiX = 0.9839) and *L. triticoides* (PiX = 1.2834). In cpDNA sequences, all *L. triticoides* were characterized by haplotypes that were in common with putative *L. cinereus* and *L. cinereus* x *L. triticoides* hybrids. ITS sequences, however, displayed haplotypes unique to

L. triticoides (haplotypes: '57', '60', '70', '71', and '72'). Haplotype '62', shared with a putative *L. cinereus* x *L. triticoides* hybrid had highest frequency (freq. = 0.2381) among *L. triticoides* accessions. Haplotype '62' is differentiated from the conglomerate haplotype '03' by one A-C transition mutation. Haplotype '03' had the second-highest frequency (freq. = 0.2143) among *L. triticoides* sequences and the highest frequency (freq. = 0.6534) among *L. cinereus* samples, demonstrating the similarity between *L. cinereus* and *L. triticoides* in the majority of accessions for the nuclear regions tested. However, significant differences were reported between *L. cinereus* and putative *L. cinereus* x *L. triticoides* hybrids ($p = 0.01802$) as well as between *L. triticoides* and putative *L. cinereus* x *L. triticoides* hybrids ($p = 0.0284$). As found in the cpDNA, a higher average number of pairwise differences were reported within accessions ($PiX = 1.5238$) of the *L. cinereus* x *L. triticoides* hybrids than when compared with *L. cinereus* ($F_{st} = 0.04127$) ($PiXY = 1.2793$) and *L. triticoides* ($F_{st} = 0.07028$) ($PiXY = 1.5000$).

As in the cpDNA analysis, *L. salinus* ssp. *mojavensis* ($PiX = 1.000$) showed fewer significant differences when compared with significant differences between other OTUs. The single *L. salinus* ssp. *mojavensis* sequence was characterized by two haplotypes, '03' and '63', separated by one A to C mutation. Several taxa that also have sequences characterized by haplotype '03' for at least one allele, had a higher number of within-average pairwise differences than when compared with *L. salinus* ssp. *mojavensis*: *L. ambiguus* ($PiX = 1.4546$) ($F_{st} = 0.00244$) ($PiXY = 1.33333$), *L. cinereus* x *L. triticoides* ($PiX = 1.52381$) ($F_{st} = 0.11173$) ($PiXY = 1.23333$), *L. salinus* ($PiX = 1.8182$) ($F_{st} = 0.04000$) ($PiXY = 1.6667$). *Leymus innovatus* was not characterized by haplotype '03',

yet it still had a higher number of within-pairwise differences ($PiX = 5.3333$) than when compared to *L. salinus ssp. mojavenensis* ($F_{st} = 0.1338$) ($PiXY = 4.5000$).

ITS phylogenies- The most parsimonious ITS phylogeny had 157 steps, a consistency index = 0.6943, and a retention index = 0.8140 (Figure 2-5). The majority of *L. cinereus* accessions (231 of 250) are characterized by haplotype '03' in at least one allelic haplotype. Eighteen *L. cinereus* haplotypes differ by only one or two steps, and haplotypes '32', '33', '34', and '59', composed of *L. cinereus* from British Columbia, Washington, and Oregon, are three to four steps different than haplotype '03'. In contrast to cpDNA, *L. triticoides* displayed more distinct groupings than *L. cinereus* in the ITS parsimony. Haplotypes '57', '58', '59', '60', '61', and '62' group together and are comprised of 19 *L. triticoides* and two *L. cinereus x L. triticoides* accessions. Haplotype '72' is composed of five *L. triticoides* accessions and groups closely with haplotype '20' comprised of 17 *L. cinereus*, one *L. triticoides* and two *L. cinereus x L. triticoides* accessions. The two accessions of the *L. triticoides* release, 'Rio', do not group specifically with other *L. triticoides* or share haplotypes with any other *Leymus* accessions. Still, the two 'Rio' haplotypes are only separated from the majority of other North American *Leymus* haplotypes by one to three steps.

Four *L. flavescens* haplotypes (from two *L. flavescens* accessions) cluster with three of the four *L. innovatus* haplotypes, about two steps from the core branch characterizing other North American *Leymus*. *Leymus flavescens* and *L. innovatus* also grouped in proximity to one another in the heuristic parsimony cpDNA phylogeny.

The remaining *L. innovatus* haplotype '78' clusters with the four *L. mollis* haplotypes and Triticeae outgroup *P. juncea*. The cpDNA neighbor-joining phylogeny

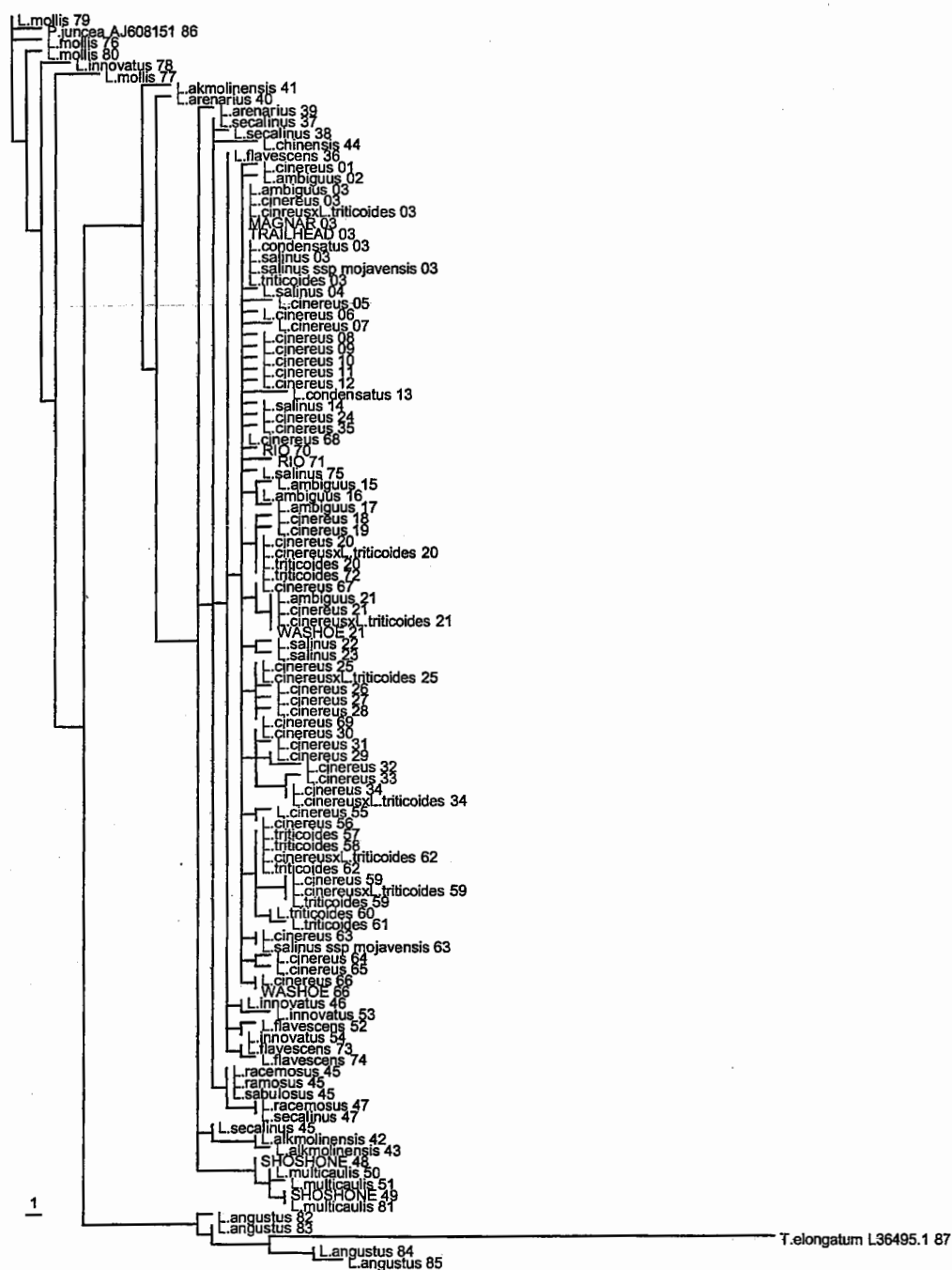


Figure 2-5. Heuristic parsimony analysis of 81 ITS haplotypes with bootstrap values determined from 500 replicates, with 157 steps, consistency index (CI= 0.6943), and retention index (RI=0.8140).

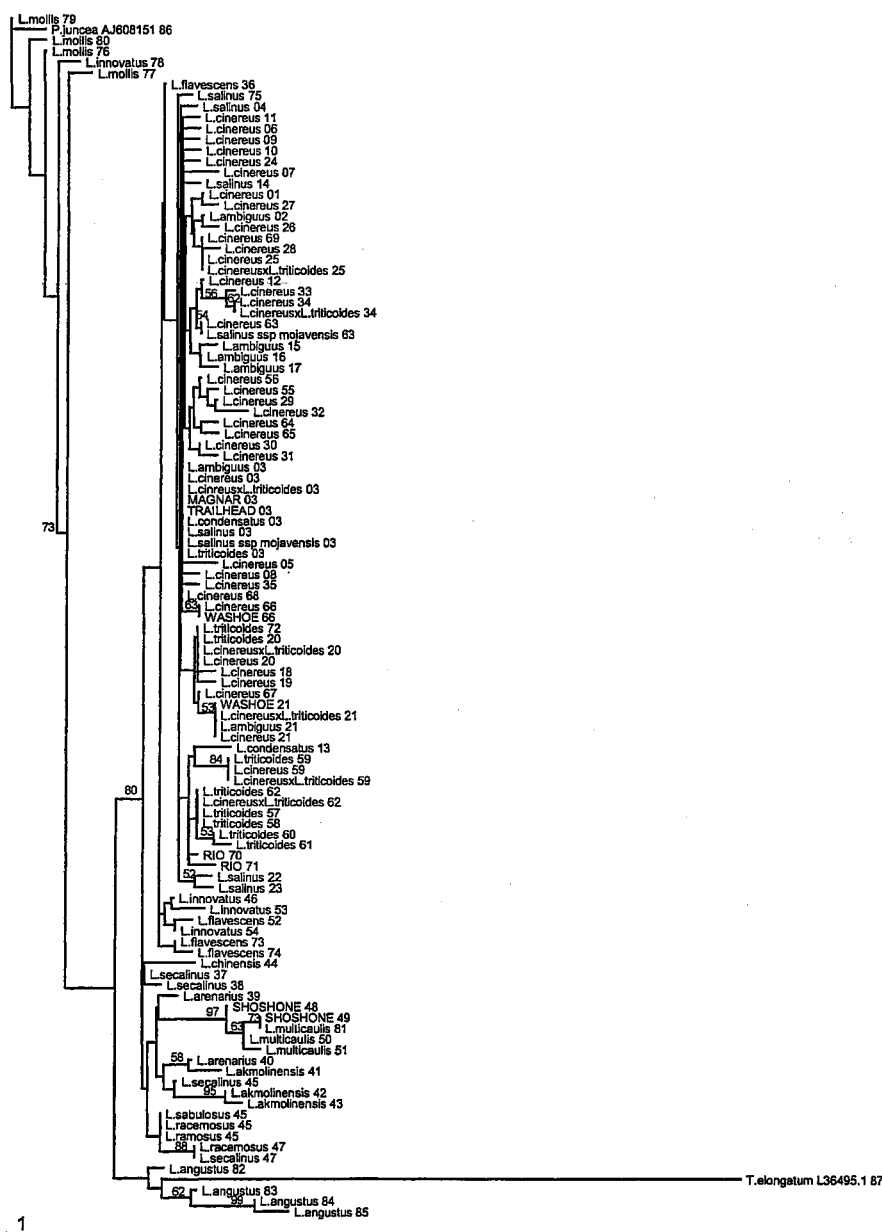


Figure 2-6. Phylogram constructed from neighbor-joining distance analysis, among 19 *Leymus* taxa and two other Triticeae taxa, based on the total number of differences (substitutions or indels) among nuclear ribosomal DNA internal transcribed spacer (ITS) haplotypes with bootstrap support values determined from 1000 sample reps.

is consistent with ITS phylogenies where *L. innovatus* groups with *L. mollis*.

Leymus mollis haplotypes, including both the European and Alaskan accessions, grouped together with *Psathyrostachys juncea*, and more distantly, but in the same clade with, *L. angustus* and *Thinopyrum elongatum* in the ITS phylogeny. *Leymus mollis* is morphologically similar to *L. arenarius* and *L. racemosus*, but prior analysis of ribosomal in situ genes (Ananthawat-Jonsson, 2001) and observations from the phylogenies in this study do not support the idea that these species have the same genetic origins. *Leymus mollis* does not group in close proximity (32 steps) to *Psathyrostachys juncea* in the cpDNA phylogenies.

Inferred haplotypes '15', '16', or '17' were present in six *L. ambiguus* accessions and cluster in the ITS parsimony and distance phylogenies. The neighbor-joining analysis clusters these *L. ambiguus* haplotypes with *L. salinus* ssp. *mojavensis* and *L. cinereus* of haplotype '63'. The three haplotypes differ by one or two steps from the main branch joining other *L. ambiguus* haplotypes, '02', '03', and '21'. There were a total of six different haplotypes among six *L. salinus* accessions. Although only two *L. salinus* haplotypes '22' and '23' group together in a unique branch, these two haplotypes include one haplotype from five of six accessions of *L. salinus*, making '22' and '23' the most common haplotypes among *L. salinus*. The remaining haplotypes are only one step different than the main branch of the clade comprising most North American *Leymus* accessions. One *L. salinus* accession is included in the conglomerate haplotype '03'. Haplotypes '04' and '75' are both comprised of one *L. salinus* accession, and haplotype '14' is comprised of two *L. salinus* accessions. *Leymus condensatus* has two haplotypes '03' and '13'. Haplotype '13' clusters with *L. triticoides* and *L. cinereus* x *L. triticoides*

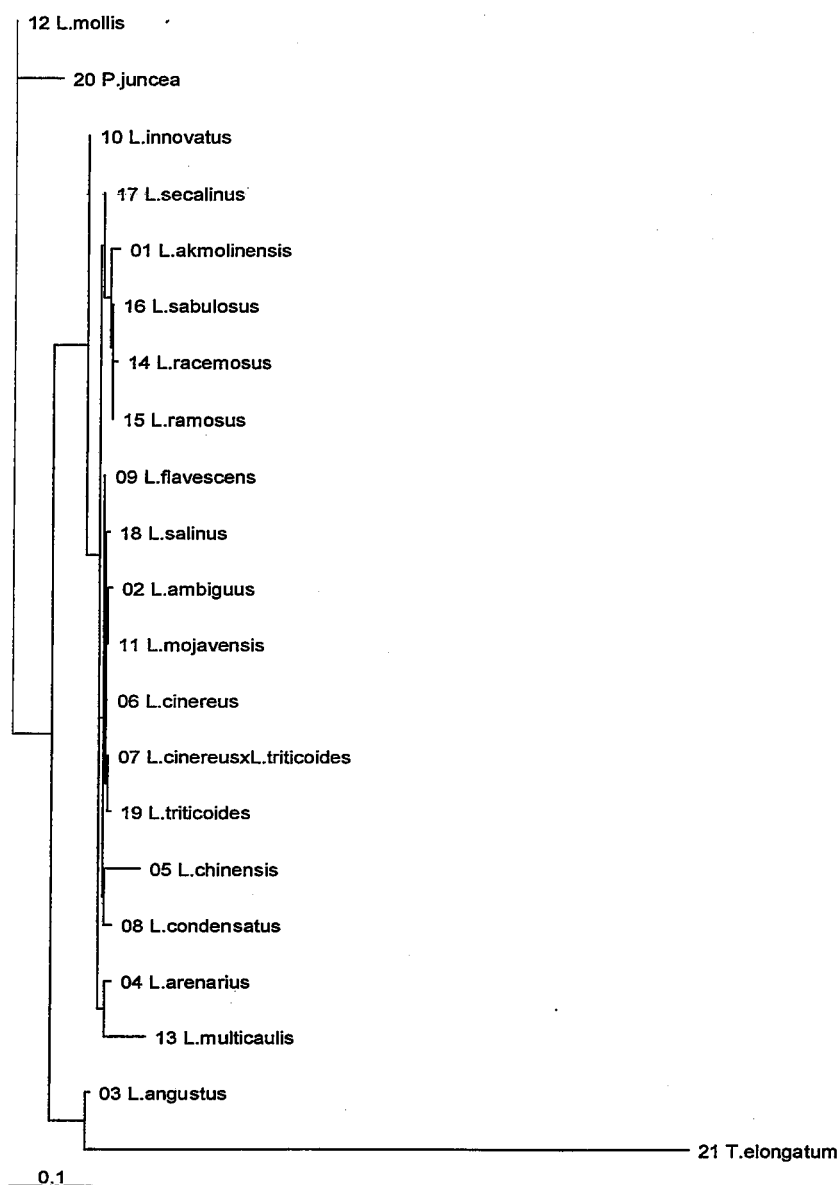


Figure 2-7. Neighbor-joining distance ITS phylogeny based on the ITS Kimura's two-parameter corrected number of average pairwise differences.

of haplotype '59' in the neighbor-joining distance phylogeny. The release, Shoshone, was first recognized as *L. triticoides* then re-identified as *L. multicaulis*. Molecular support to the later identification was found when sequences from the Shoshone release claded with

L. multicaulis in the ITS phylogeny (Figure 2-5, 2-6). Washoe Germplasm, another release, has two haplotypes '21' and '66' for replicates of the same accession. Three steps separate the two Washoe haplotypes in the heuristic phylogeny.

The two Triticeae outgroups, *Thinopyrum elongatum* and *Psathyrostachys juncea*, displayed inconsistent relationships with *Leymus* taxa between cpDNA and ITS data. The cpDNA NJ phylogeny based on K2P-corrected average pairwise differences among species makes a clear distinction between North American and Eurasian *Leymus* species. The ITS NJ phylogeny makes little distinction between North American and Eurasian taxa (Figure 2-7). *Thinopyrum elongatum* was separated from the main stem of North American *Leymus* by 11 steps in the cpDNA phylogeny, where in the ITS phylogeny it was 38 steps from the closest North American taxon. In contrast, *Psathyrostachys juncea* was 31 steps from the closest North American taxon in cpDNA, where it was only two steps different from the closest North American taxon in the ITS phylogeny.

Paired sites- Of particular interest to researchers is the capacity of *L. cinereus* and *L. triticoides* to hybridize. Several localities that included both *L. cinereus* and *L. triticoides* were sampled additionally to verify gene flow between different species in proximity to each other. Chloroplast DNA haplotype '37' characterized samples CIN_OR003 (*L. cinereus*) and TRI_OR008 (*L. triticoides*) collected from the same locality. Haplotype '47' is common between 'CIN_NV056' (*L. cinereus*) and 'TRI_NV003' (*L. triticoides*) also from the same locality. The accessions characterized by cpDNA haplotype '37' and '47' also grouped in the assorted ITS haplotype '03' (Table 2-2). Other paired sites, 'TRI_NV007' and 'CIN_NV006', as well as

'PAR_OR001' and 'TRI_OR007', were characterized by haplotype '44'. Haplotype '44' distinguished 28 additional accessions including 19 *L. cinereus*, and nine *L. triticoides* collected in California, Idaho, Nevada, and Oregon.

Genetic and geographic distance correlation- The correlation between genetic distance and geographic distance for 247 *Leymus cinereus* accessions was tested for both cpDNA and the ITS Phase B data. The matrix correlation between sites for cpDNA was non-significant, $r = 0.008$ ($p = 0.349$). A significant difference was reported for the correlation between sites in ITS phase B, $r = 0.15726$ ($p = 0.002$).

DISCUSSION

The primary objective of this research project was to determine if selected cpDNA and nuclear loci could serve as efficient barcoding tools to identify species. Secondly, nuclear and chloroplast DNA phylogenies were constructed for 319 *Leymus* wildryes. Thirdly analyses, were conducted to see if a correlation exists between genetic distance and geographic provenance among cultivated and natural North American *L. cinereus* accessions.

Analysis of molecular variance F-statistics values and comparisons of average pairwise differences were used to test the effectiveness of DNA barcodes to detect informative polymorphisms unique to a species. Chloroplast DNA *trnH-psbA* and *trnK-rps16* sequences and ITS sequences differentiated North American taxa *L. flavescens*, *L. innovatus*, and *L. mollis* with unique polymorphic loci, but none were significantly different from all the other *Leymus* based on average pairwise differences. The most frequent cpDNA haplotype unique to *L. cinereus*, '40', was only two transition mutations

different from haplotypes, '41' and '44', that characterized *L. cinereus*, *L. triticoides*, and putative hybrids with the greatest frequency. When comparing cpDNA sequences, a pattern in significant differences was observed between *L. ambiguus*, *L. cinereus*, *L. cinereus x L. triticoides*, *L. salinus*, and *L. triticoides* and the other OTUs tested. Chloroplast DNA data revealed a high number of total pairwise differences within species in proportion to total pairwise differences between species in several taxa (*L. cinereus x L. triticoides*, *L. multicaulis*, *L. racemosus*, *L. salinus*, *L. secalinus*, and *L. triticoides*).

Leymus salinus, the taxon with the highest number of within-species differences, appears in three separate clades in parsimony and distance topologies. The high level of within-pairwise differences may put the identity of some of the accessions in question or suggests hybridization or introgression between taxa, making it difficult to establish distinctive haplotypes for a specific species.

Internal transcribed spacer barcodes showed more distinguishing characters among OTUs. In the ITS data, several species (*L. ambiguus*, *L. angustus*, *L. cinereus*, *L. cinereus x L. triticoides*, *L. salinus*, and *L. triticoides*) are significantly different from all other OTUs except *L. salinus ssp. mojavenensis*. Of all *Leymus* sampled with ITS barcoding primers, only three species, all from middle Asia (*L. angustus*, *L. multicaulis*, and *L. secalinus*), were significantly different from *L. salinus ssp. mojavenensis*. This can probably be largely attributed to the fact that there was only one *L. salinus ssp. mojavenensis* in the analysis. Chloroplast DNA showed a lack of significant difference between *L. salinus ssp. mojavenensis* and all other taxa with the exception of *L. racemosus*, *L. cinereus x L. triticoides*, and *L. triticoides*. This is not a pattern observed in other *L. salinus* accessions.

Despite these differences, a preponderance of non-significance between OTUs suggests the combined chloroplast *trnH-psbA* and, *trnK-rps16* and ITS nuclear barcoding regions were not sufficient in distinguishing specimens to a species-specific level.

The percentage of informative characters based on all polymorphic characters in cpDNA sequences of 19 *Leymus* species and 319 accessions examined was 6.9% (88 bp out of 1275 bp aligned characters in cpDNA), a seemingly reasonable percentage. Yet a high proportion of these polymorphic characters can be attributed to differences between Eurasian and North American taxon, rather than differences within North American subgroups, which comprise the majority of accessions sampled. Analysis of molecular variance analyses attributed 84.53 % of the variation to differences between North American and Eurasian taxa, where only 7.46% of the variation was attributed to differences within North American and Eurasian *Leymus* groups. Within species variation (8.01%) was very similar to within group differences. Chloroplast DNA phylogenies illustrate the distinct differences between North American and Eurasian *Leymus* species. Molecular divergence estimates between North American and Eurasian *Leymus* range from 650 000 to 2.3×10^6 mya.

The ITS data attributed a lower percentage (46.79%) of overall differences to comparisons between North American and Eurasian outgroups. Percent variation within North American and Eurasian groups (31.64% variation) and within species (21.58%) was higher than what was reported for the cpDNA. Additionally, an ITS retention index value of 0.8140 suggests a high proportion of polymorphisms were autapomorphic.

Many evolutionary processes at the organismal as well as molecular level have the ability to confound phylogenetic inference. There can be many reasons for

incongruence, for example, lineage sorting, introgression, and reticulation (Wendel and Doyle, 1998; Sang and Zhang, 2000). Still, incongruencies between cpDNA and nuclear data are consistent with the findings of other Triticeae species (Mason-Gamer et al., 1995; Redinbaugh et al., 2000). Inconsistencies between species designations were observed when considering differences between the ITS and cpDNA NJ phylogenies (Figure 3 and 5) derived from AMOVA average number of pairwise differences.

Chloroplast DNA sequences group *L. mollis* in proximity to the North American clades, where ITS sequences distinctly group *L. mollis* with *Psathyrostachys juncea*. *Leymus triticoides* and *L. cinereus* cluster with the hybrid *L. cinereus* x *L. triticoides* in the ITS. Chloroplast DNA groups *L. triticoides* with *L. condensatus*, and the *L. cinereus* x *L. triticoides* hybrid is closer to *L. cinereus*.

Direct observations reveal some congruence and some incongruence between ITS and cpDNA distance and parsimony phylogenies. It was difficult to make a direct comparison, as the ITS sequences had many heterozygous polymorphisms, requiring the inference of haplotypes. There were few equivalent relationships among North American *Leymus* accessions observed in cpDNA and ITS analyses. On the whole, distinctions between Eurasian outgroups and North American *Leymus* taxa are more readily observed in the cpDNA phylogenies, whereas in the ITS phylogenies, Eurasian outgroups cluster within a few steps of the North American taxa. CpDNA sequences form somewhat distinguishable clades within North American taxa, where ITS haplotypes from shallow clusters, only one or two steps from the main branch from which all North American accessions stem.

The average number of pairwise differences between Triticeae reference sequences, *T. elongatum* and *T. ponticum* and the closest North American *Leymus* taxa (*L. innovatus*) was (PiXY=6.0000). The average number of pairwise differences between *T. elongatum* and the closest haplotype containing North American *Leymus* taxa (*L. mollis*) was (PiXY= 37.5000) in ITS sequences. The close relationship of *T. elongatum* (eight nucleotide differences) to North American *Leymus* taxa in the cpDNA analysis may revive speculation that *Thinopyrum* is the other diploid ancestor of North American *Leymus*. However further experimentation with the *trnH-psbA* and *trnk-rps16* regions and other Triticeae taxa will be necessary to make a proper assessment about the origins of the *Leymus* genome. Chloroplast DNA also illustrates a close relationship between Eurasian *Leymus* and *Psathyrostachys juncea*, one of the diploid ancestors of *Leymus*.

Psathyrostachys juncea shows a closer relationship to North American *Leymus* in the ITS data. The average number of pairwise differences between *P. juncea* and *L. innovatus* is greater in the cpDNA (PiXY= 30.0000) than when *P. juncea* is compared to *L. innovatus* in the ITS (PiXY = 7.8333). The ITS parsimony analyses, based on 85 *Leymus* haplotypes, shows *L. innovatus* and *L. flavescentis* are the most closely related descendants of the *Leymus* outgroup clades, while *L. mollis* groups with *P. juncea*. The cpDNA heuristic parsimony analysis, in contrast, does not outline a distinct relationship between *L. mollis* and *P. juncea*. However, *L. mollis* is the closest descendant of all of the outgroup reference taxa, followed by *L. innovatus* and *L. cinereus* haplotypes '20' and '37'.

A portion of the research for this project focused on the putative hybrids between *L. cinereus* and *L. triticoides*. Several of the designated 'paired sites' resulted in the same

cpDNA haplotype, indicating admixture between the accessions. Comparisons of the total average number of pairwise differences within the hybrids were higher ($PiX = 3.7222$) than between the hybrids and *L. cinereus* ($F_{st} = 0.0784$) ($PiXY = 3.04530$) and *L. triticoides* ($F_{st} = 0.0735$) ($PiXY = 2.6455$) in the cpDNA. Similarly, ITS sequences reported a higher average number of pairwise differences within accessions ($PiX = 1.5238$) of the hybrids than when compared with *L. cinereus* ($F_{st} = 0.04127$) ($PiXY = 1.2793$) and *L. triticoides* ($F_{st} = 0.07028$) ($PiXY = 1.5000$). A higher number of average pairwise differences within the hybrids than between hybrids and other taxa may indicate varying degrees of parental influence from either *L. cinereus* or *L. triticoides* among different accessions. There was a significant difference ($p \leq 0.05$) between the putative *L. cinereus* x *L. triticoides* hybrid accessions and *L. triticoides* in the cpDNA analysis. Significant differences ($p \leq 0.05$) were also observed in all comparisons between *L. cinereus*, *L. triticoides* and the putative *L. cinereus* x *L. triticoides* hybrid accessions in ITS sequences. Neither cpDNA nor ITS phylogenies generated any obvious clusters to suggest a tendency for hybrids to group with one of the parental taxa more than the other. A more equitable representation of taxa, additional sequences from other cpDNA and nuclear genome regions, as well as more robust molecular techniques, may provide more insight into evaluating possible hybridization patterns in *Leymus*.

There appears to be little discernable grouping among OTUs related to species designations or geography. Studies with wide-ranging geographical sampling (Mason-Gamer et al., 1995; Larson, et al., 2003b) have found a high degree of inter and intraspecific variation within species in correlation to geographical distance (Comes and Abbott, 2001). However, in our analyses correlations between geographic and genetic

distance have been less than 30%. Some possible contributing factors include the possibility of an unknown degree of introgression or hybridization in the samples tested. Yet another possibility is an unknown level of historical and/or contemporary distribution of plant seed via anthropogenic movement or other mechanisms.

A study by Yang et al. (2006) constructed a dendrogram of 20 *Leymus* species and 40 accessions based on 192 random amplified microsatellite polymorphisms from 24 primer combinations. Accessions of the same species generally clustered together. A direct comparison of topologies between studies was hampered due to a higher proportion of Eurasian accessions in comparison to our study, and only six accessions (*L. ambiguus*: PI531795, MAGNAR: PI469229, TRAILHEAD: PI478831, *L. cinereus*: PI537353, *L. cinereus x L. triticoides*: PI537363, *L. ramosus*: PI499653) were in common between the two analyses.

According to Chase et al. (2005), “more sophisticated barcoding tools, such as multiple low-copy nuclear markers with sufficient genetic variability and PCR-reliability, would permit the detection of hybrids and permit researchers to identify the ‘genetic gaps’ that are useful in assessing species limits.” An alternative to barcodes may be the use of increasingly available nuclear microsatellites, which are generally unlinked and codominant, and have a broad taxonomic coverage and high levels of polymorphisms (Zane et al., 2002; Duminil et al., 2006).

LITERATURE CITED

- ANAMTHAWAT-JONSSON, K., 2001. Genetic and genomic relationships in *Leymus* Hochst. *Hereditas* 135: 247-253.

- ANAMTHAWAT-JONSSON, K., AND S. K. BODVARSDOTTIR. 1998. Meiosis of wheat x lymegrass hybrids *Chromosome research* 6: 339-343.
- ANAMTHAWAT-JONSSON, K., AND S. K. BODVARSDOTTIR. 2001. Genomic and genetic relationships among species of *Leymus* (Poaceae: Triticeae) inferred from 18S-26S ribosomal genes. *American Journal of Botany* 88: 553-559.
- BAILEY, R. G., AND C. T. CUSHWA. 1981. Ecoregions of North America (map). (FWS/OBS-81/29.) Washington, DC: U.S. Fish and Wildlife Service. 1:12,000,000. Washington, DC, USA.
- BALDWIN, B. G. 1992. Phylogenetic utility of the internal transcribed spacers of nuclear ribosomal DNA in plants: an example from the Compositae. *Molecular Phylogenetics and Evolution* 1:3-16.
- BALDWIN, B. G. 1995. The ITS region of nuclear ribosomal DNA- A valuable source of evidence on angiosperm phylogeny. *Annals of the Missouri Botanical Garden* 82 2: 247.
- BARKWORTH, M. E., 2007. *Leymus* Hochst. In M. E. Barkworth, K. M. Capels, S. Long, L. K. Anderton, and M. B. Piep [eds.], Flora of North America North of Mexico, Volume 24. Magnoliophyta: Commelinidae (in part): Poaceae, part 1. pp. 353-369. Oxford University Press, Inc. New York, New York, USA.
- BARKWORTH, M. E., AND R. J. ATKINS. 1984. *Leymus* Hochst (Gramineae, Triticeae) in North American Taxonomy and distribution. *American Journal of Botany* 71 5: 609-625.
- BARKWORTH, M. E., AND S. W. L. JACOBS. 2001. Valuable research or short stories: What makes the difference? *Hereditas* 135: 263-270.
- BENTHAM, G. 1881. Notes on Gramineae. *Botanical Journal of Linnean Society*. London 19: 14-134.
- BODVARSDOTTIR, S. K., AND K. ANAMTHAWAT-JONSSON. 2003. Isolation, characterization, and analysis of *Leymus*-specific DNA sequences. *Genome* 46: 673-682.
- BOWDEN, W. M. 1957. Cytotaxonomy of section *Psammelymus* of the genus *Elymus*. *Canadian Journal of Botany* 35: 951-993.
- BOWDEN, W. M. 1964. Cytotaxonomy of the species and interspecific hybrids of the genus *Elymus* in Canada and neighboring areas. *Canadian Journal of Botany* 42: 547-601.

- BOWDEN, W. M. 1967. Taxonomy of intergeneric hybrids of the tribe Triticeae from North America. *Canadian Journal of Botany* 45: 711-724.
- CHARMET G., C. RAVEL, AND F. BALFOURIER. 1997. Phylogenetic analysis in the *Festuca-Lolium* complex using molecular markers in ITS rDNA. *Theoretical and Applied Genetics* 94: 1038-1046.
- CHASE, M. W., N. SALAMIN, AND M. WILKINSON. 2005. Land plants and DNA barcodes: short-term and long-term goals. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 360: 1889-1895.
- COMES, H. P., AND R. J. ABBOTT. 2001. Molecular phylogeography, reticulation, and lineage sorting in Mediterranean *Senecio* sect. *Senecio* (Astraceae). *Evolution* 55: 1943-1962.
- COWAN, R. S., M. W. CHASE, J. W. KRESS, AND V. SAVOLAINEN. 2006. 300,000 species to identify: problems, progress, and prospects in DNA barcoding of land plants. *Taxon* 55: 611-616.
- CRONQUIST, A. H., H. ARTHUR, AND N. H. HOLMGREN. 1977. Intermountain flora: Vascular plants of the Intermountain West, U.S.A. New York. Columbia University Press, New York, New York, USA.
- DEWEY, D. R. 1972. Cytogenetic and genomic relationships of *Elymus giganteus* and *E. dasystachys* and *E. junceus*. *Bull Torrey Botanic Club* 99:77-83.
- DEWEY, D. R. 1984. The genomic system of classification as a guide to intergeneric hybridization with the perennial Triticeae. In J. P. Gustafson [eds.], Proceedings of the 16th Stadler Genetics Symposium, Columbia, Missouri, 19-21 March. pp. 209-279. Plenum, New York, New York, USA.
- DUMINIL, J., H. CARON, I. SCOTTI, S. O. CAZAL, AND R. J. PETIT. 2006. Blind population genetics survey of tropical rainforest trees. *Molecular Ecology* 15: 3505-3513.
- EXCOFFIER, L., P. E. SMOUSE, AND J. M. QUATTRO. 1992. Analysis of variance inferred from metric distances among DNA haplotypes: applications to human mitochondrial DNA restriction data. *Genetics* 131: 479-491.
- FJELLHEIM, S., O. A. ROGNLI, K. FOSNES, AND C. BROCHMANN. 2006. Phylogeographical history of the widespread meadow fescue (*Festuca pratensis* Huds.) inferred from chloroplast DNA sequences. *Journal of Biogeography* 33: 1470-1478.

- FERGUSON, R. B., AND N. C. FRISCHKNECHT. 1985. Reclamation on Utah's Emery and Alton coal fields: techniques and plant materials. Res. Pap. INT-335. Ogden, UT: U.S. Department of Agriculture, Forest Service, Intermountain Forest and Range Experiment Station.
- HICKMAN, J. C. 1993. The Jepson manual: higher plants of California. Berkeley, CA: University of California Press, Berkeley, California, USA.
- HITCHCOCK, A. S. 1935. Manual of the grasses of the United States. USDA Misc. Publ. No. 200.
- HITCHCOCK, A. S. 1951. Manual of the grasses of the United States 2nd ed., Revised by A. Chase. USDA misc. Publ. No. 200.
- HITCHCOCK, C. L., A. CRONQUIST, AND M. OWNBEY. 1969. Vascular plants of the Pacific Northwest. Part 1: Vascular cryptograms, gymnosperms, and monocotyledons. University of Washington Press, Seattle, Washington, USA.
- HOLE, D. J., AND K. B. JENSEN. 1999. Molecular marker analysis of *Leymus flavescens* and chromosome pairing in *Leymus flavescens* hybrids (Poaceae: Triticeae). *International Journal of Plant Sciences* 160: 371-376.
- JONES, T. A., AND D. A. JOHNSON. 1998. Integrating genetic concepts into planning rangeland seedings. *Journal Range Management* 51: 594-606.
- JONES, T. A., D. C. NIELSON, J. T. ARRENDONDO, AND M. G. REDINBAUGH. 2003. Characterization of diversity among 3 squirreltail taxa. *Journal of Range Management* 56: 474-482.
- KIMURA, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16, 111-120.
- KIMURA, M. 1981. Estimation of evolutionary distances between homologous nucleotide sequences. *Proceeding of the national Academy of Sciences of the United States of America*. 78: 454-458.
- KRESS J. W., K. J. WURDACK, E. A. ZIMMER, L. A. WEIGT, AND D. H. JANZEN. 2005. Use of DNA barcodes to identify flowering plants. *Proceeding of the National Academy of Sciences of the United States of America* 102: 8369-8374.
- KUMAR, S., K. TAMURA, AND M. NEI. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics* 5: 150-163.

- LARSON, S. R., T. A. JONES, Z. M. HU, A. PALAZZO, AND C. L. MCCrackEN. 2000. Genetic diversity of bluebunch wheatgrass cultivars and a multiple-origin polycross. *Crop Science* 40: 1142-1147.
- LARSON, S. R., T. A. JONES, AND K. B. JENSEN. 2004 Population structure of *pseudorenaria spicata* (Poaceae: Triticeae) modeled by Bayesian clustering of AFLP genotypes *American Journal of Botany* 91: 1789-1801.
- LARSON, S. R., T. A. JONES, C. L. MCCrackEN, AND K. B. JENSEN. 2003a. Amplified Fragment length polymorphism in *Elymus elymoides*, *Elymus multisetus*, and other *Elymus* taxa. *Canadian Journal of Botany* 81: 789-804.
- LARSON, S. R., A. J. PALAZZO, AND K. B. JENSEN. 2003b. Identification of western wheatgrass cultivars and accessions by DNA fingerprinting and geographic provenance. *Crop Science* 43: 394-401.
- MANTEL, N. A. 1967. The detection of disease clustering and a generalized regression approach. *Cancer Research* 27: 209-220.
- MARTY, L. J. 2002. Development of acid/heavy metal-tolerant cultivars. Bridger Plant Materials Center, Route 2, Box 1 189, Bridger, Montana, USA.
- MASON-GAMER, R. J., K. E. HOLSINGER, AND R. K. JANSEN. 1995. Chloroplast DNA haplotype variation within and among populations of *Coreopsis grandiflora* (Asteraceae). *Molecular Biology Evolution*. 12: 371-381.
- MCKENZIE, R. J., E. M. MULLER, A. K. W. SKINNER, P. O. KARIS, AND N. P. BARKER. 2006. Phylogenetic relationships and generic delimitation in subtribe Arctotidinae (Asteraceae: Arctotideae) Inferred by DNA sequence data from ITS and five chloroplast regions. *American Journal of Botany* 93: 1222-1235.
- NEVESKI, C. A. 1933. Agrostologische Studien IV. Über das System tribe Hordeae Benth. *Trudy Botanicheskogo Instituta Akademii Nauk SSSR. Ser. 2. Sporovye Rasteniya* 1: 9-32.
- OGIHARA, Y., T. TERACHI, AND T. SASAKUMA. 1991. Molecular analysis of the hot-spot region related to length mutations in wheat chloroplast DNAs I. nucleotide divergence of genes and intergenic spacer regions located in the hot-spot region. *Genetics* 129: 873-884.
- OGLE D. G., L. K. HOLZWORTH, S. R. WINSLOW, AND T. A. JONES. 2003. Basin Wildrye *Leymus cinereus* (Scribn. & Merr.) A. Love plant symbol = LECI4, USDA NRCS Idaho State Office, Montana State Office, Aberdeen Plant Materials Center, Montana Plant Materials Center, & the National Plant Data Center; USDA ARS Forage & Range Laboratory.

- PILGER, R. 1949. Additamenta agrostologica. I. Triticeae (Hordeae). *Botanische Jahrbücher für Systematik* 74: 1-13.
- REDINBAUGH, M. G., T. A. JONES, AND Y. ZHANG. 2000. Ubiquity of the St chloroplast genome in St-containing Triticeae polyploids. *Genome* 43: 846-852.
- RICHARDS, R. T., J. C. CHAMBERS, AND C. ROSS. 1998. Use of native plants on federal lands: policy and practice. *Journal of Range Management* 51: 625-632.
- ROHLF, F. J. 1998. NTSYS-pc: numerical taxonomy and multivariate analysis system version 2.0. Exeter Software, Setauket, New York, USA.
- SAITOU, N., AND M. NEI. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biological Evolution* 4: 406-425.
- SANG, T., AND Y. ZHONG. 2000. Testing hybridization hypotheses based on incongruent gene trees. *Systematic Biology* 49: 422-434.
- SHAW J., E. B. LICKY, J. T. BECK, S. B. FARMER, W. S. LIU, J. MILLER, K. C. SIRIPUN, C. T. WINDER, E. E. SCHILLING, AND R. L. SMALL. 2005. The tortoise and the hare II: Relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *American Journal of Botany* 92: 142-166.
- SIMMONS, M. P., AND H. OCHOTERENA. 2000. Gaps as characters in sequence-based phylogenetic analyses. *Systematic Biology* 49: 369-381.
- SMOUSE, P. E., LONG, J. C., SOKAL, R. R. 1986. Multiple-regression and correlation extensions of the Mantel test of matrix correspondence. *Systematic Zoology* 35: 627-632.
- SOLTIS, D. E., L. A. JOHNSON, AND D. C. LOONEY. 1996. Discordance between ITS and chloroplast topologies in the *Boykinia* group (Saxifragaceae). *Systematic Botany* 21: 169-185.
- STEBBINS, G. L. 1981. Co-evolution of grasses and herbivores. *Annals of Missouri Botanical Garden* 68: 75-86.
- STEPHENS, M., AND P. DONNELLY. 2003. A comparison of bayesian methods for haplotype reconstruction. *American Journal of Human Genetics* 73: 1162-1169.
- STEPHENS, M., N. J. SMITH, AND P. DONNELLY. 2001. A new statistical method for haplotype reconstruction from population data. *American Journal of Human Genetics* 68: 978-989.

- SWOFFORD, D. L. 2000. PAUP* Phylogenetic analysis using parsimony (*and other methods). Version 4.0b10. Sinaur Associates, Sunderland, Massachusetts, USA.
- WANG, R. R. -C., AND K. B. JENSEN. 1994. Absence of the J genome in *Leymus* species (Poaceae: Triticeae): evidence from DNA hybridization and meiotic pairing. *Genome* 37: 231-235.
- WANG, R. R. -C., R. VON BOTHMER, J. DVORAK, I. LINDE-LAURSEN, AND M. MURAMATSU. 1994. Genome symbols in the Triticeae (Poaceae). In Proceedings of the 2nd international Triticeae Symposium, Logan, Utah, 20–24 June. Edited by R. R. -C. Wang, Jensen K. B., and Jaussi, C. pp. 29-31. Utah State University Press, Logan, Utah, USA.
- WASSER, C. H. 1982. Ecology and culture of selected species useful in revegetating disturbed lands in the West. FWS/OBS-82/56. U.S. Department of the Interior, Fish and Wildlife Service, Office of Biological Services, Western Energy and Land Use Team, Washington, DC, USA..
- WENDEL, F. W., AND J. J. DOYLE. 1998. Phylogenetic incongruence: window into genome history and molecular evolution. In D. E. Soltis, P. S. Soltis, and J. J. Doyle, [eds.], Molecular systematics of plants. II. DNA sequencing. 265– 296. Kluwer Academic Publishers, Boston, Massachusetts, USA.
- WHITE, T. J., T. BRUNS, S. LEE, AND J. TAYLOR. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In Innis, M., D. Gelfand, J. Sninsky, and T. White [eds.], PCR Protocols: A Guide to Methods and Applications, pp. 315-322. Academic Press, San Diego, California, USA.
- WU, X., S. L. LARSON, H. ZANMIN, P. J. ANTONIO, T. A. JONES, R. R. -C. WANG, K. B. JENSEN, AND J. N. CHATTERTON. 2003. Molecular Genetic linkage maps for allotetraploid *Leymus* Wildryes (Gramineae: Triticeae). *Genome* 46: 627-646.
- YANG, R., Y. H. ZHOU, Y. ZHANG, Y. L. ZHENG, AND C. B. DING. 2006. The genetic diversity among *Leymus* species based on random amplified microsatellite polymorphism (RAMP). *Genetic Resources and Crop Evolution* 53: 139-144.
- ZANE, L., L. BARGELLONI, AND T. PATARNELLO. 2002. Strategies for microsatellite isolation: a review. *Molecular Ecology* 11: 1–16.
- ZHANG, H.B., AND J. DVORAK. 1991. The genome origin of tetraploid species of *Leymus* (Poaceae: Triticeae) inferred from variation in repeated nucleotide sequences. *American Journal of Botany* 78: 871-884.
- ZHANG, D., AND G. M. HEWITT. 2003. Nuclear DNA analyses in genetic studies of populations: practice, problems and prospects. *Molecular Ecology* 12: 563-584.

APPENDIX A

MORPHOLOGY

Leymus species are perennial, caespitose or rhizomatous, and hermaphroditic. Spike growth, color, forage yield, and germination percentage are highly variable (Cronquist et al., 1977). Culms are erect or ascending 10-350 cm, with terete, hollow, glabrous, and either scabrous or hairy internodes. Leaves are basal and cauline, with blades that are flat, involute, or linear, either stiff or lax. The leaf veins are either equally spaced prominently ribbed veins or unequal, widely spaced, and not prominent. The inflorescence is termed a “spike” despite spikelets on pedicels up to 2 mm long. Disarticulation is above the glumes and beneath the florets. Spikelets are usually paired, with sometimes up to eight pediceled spikelets per node and two to twelve florets per spikelet. The glumes are equal to subequal, lanceolate to subulate, narrowing in the distal ¼ or tapering from below midlength, conspicuously keeled, and opposite the sides of the lemmas rather than the midveins. The glumes and lemmas are glabrous to pilose, with mostly unawned or short awned apices. The paleas are subequal to the lemmas. There are two lodicules that have either short hairs or are lobed. The three anthers are 2.5 to 10 mm long. The caryopses are hairy at the apices (Nevski, 1933; Barkworth and Atkins, 1984; Barkworth, 2007).

APPENDIX B

SEQUENCING PCR METHODS

Amplification of DNA sequences for ITS primers were performed with Fermentas reagents. The 25- μ l volume reactions included 2.5- μ l 20mM 10x Taq Buffer with KCl, 1.5- μ l 25 mM MgCL₂, 2.5 μ l 2mM dNTP, 0.4 μ M primers, 1 unit *Taq* DNAP, and approximately 40 ng of plant DNA using the following temperature profile: 94° C (2.5 min); 35 cycles of 94° C denaturing (20 sec), 54° C annealing (30 sec), and 72° C extension (2 min); 72° C extension (7 min).

Amplification of chloroplast DNA sequences was performed using Fermentas and Promega reagents in 25- μ l volume reactions: 2.5- μ l 20mM 10x Taq Buffer with KCl, 2.0- μ l 25 mM MgCL₂, 2.5- μ l 2mM dNTP 0.4 μ M primers, 1 unit *Taq* DNAP, and approximately 30 ng of plant DNA using the following temperature profile: 94° C (1 min); 5 cycles of 94° C denaturing (30 sec), 53° C annealing (45 sec), and 72° C extension (1.5 min); 30 cycles of 94° C denaturing (30 sec), 48° C annealing (45 sec), and 72° C extension (1 min); 72° C extension (7 min).

CHARACTERIZATION OF cpDNA ALLELIC HAPLOTYPES

60

APPENDIX D

CHLOROPLAST DNA TOTAL NUMBER OF AVERAGE PAIRWISE DIFFERENCES

Appendix D. cpDNA Total average number of pairwise differences within (diagonal), between (above diagonal) and corrected average pairwise differences between (below diagonal) 18 *Lemna* and four *Trifolium* taxa

OTU species name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1: <i>L. microlophus</i>	1,000	32,500	1,500	1,500	3,000	29,879	30,300	30,500	31,500	27,500	29,500	30,500	2,500	2,500	0,500	1,500	2,500	30,833	30,167	29,500	28,500	7,500	7,500
2: <i>L. gibbus</i>	30,600	2,800	33,000	33,000	23,500	8,000	8,000	8,000	8,000	8,000	8,000	8,000	33,000	32,400	32,000	33,000	32,000	8,000	8,000	11,000	10,000	24,000	24,000
3: <i>L. perfoliatus</i>	1,000	31,600	0,000	2,000	3,000	30,379	30,800	31,000	32,000	28,000	29,000	30,000	2,500	1,000	1,000	0,000	3,000	31,333	30,667	30,000	29,000	8,000	8,000
4: <i>L. reniformis</i>	1,000	31,600	2,000	0,000	3,000	30,379	30,800	31,000	32,000	28,000	29,000	30,000	2,500	3,000	1,000	0,000	3,000	31,333	30,667	30,000	29,000	8,000	8,000
5: <i>L. chinensis</i>	2,000	27,600	3,000	3,000	1,000	26,879	27,300	27,500	28,500	24,500	25,500	26,500	4,000	3,000	2,000	2,000	1,500	21,833	21,167	20,500	19,500	5,500	5,500
6: <i>L. chinensis</i>	28,258	6,010	29,258	29,258	25,258	2,826	2,826	2,826	2,826	3,078	3,078	3,078	30,678	30,678	30,678	30,678	27,778	6,833	6,833	8,078	8,078	31,850	32,378
7: <i>L. chinensis x trifolium</i>	28,087	5,233	29,087	29,087	25,087	0,022	0,022	0,022	0,022	3,200	3,200	3,200	30,600	30,600	30,600	30,600	28,200	6,433	6,433	7,524	7,524	31,850	32,378
8: <i>L. condensatus</i>	31,000	4,600	31,000	31,000	27,000	0,353	0,353	0,353	0,353	4,000	4,000	4,000	30,500	30,500	30,500	30,500	28,400	6,887	6,887	8,000	8,000	32,000	32,000
9: <i>L. hayesii</i>	27,000	4,433	28,000	28,000	24,000	1,828	1,828	1,828	1,828	4,000	4,000	4,000	30,500	30,500	30,500	30,500	28,400	6,887	6,887	8,000	8,000	32,000	32,000
10: <i>L. henricus</i>	29,000	4,433	30,000	30,000	26,000	3,753	3,753	3,753	3,753	4,000	4,000	4,000	30,500	30,500	30,500	30,500	28,400	6,887	6,887	8,000	8,000	32,000	32,000
11: <i>L. salinus</i>	30,000	7,433	31,000	31,000	27,000	4,957	4,957	4,957	4,957	5,000	5,000	5,000	30,500	30,500	30,500	30,500	28,400	6,887	6,887	8,000	8,000	32,000	32,000
12: <i>L. moenchii</i>	30,000	7,433	31,000	31,000	27,000	4,957	4,957	4,957	4,957	5,000	5,000	5,000	30,500	30,500	30,500	30,500	28,400	6,887	6,887	8,000	8,000	32,000	32,000
13: <i>L. microlophus</i>	1,333	30,600	0,333	2,333	2,000	28,258	28,680	28,880	29,000	27,000	28,000	29,000	1,833	1,333	0,000	1,000	2,800	30,333	29,667	29,000	28,000	7,887	7,887
14: <i>L. ramosus</i>	0,000	30,600	1,000	1,000	2,000	28,258	28,680	28,880	29,000	27,000	28,000	29,000	1,833	1,333	0,000	1,000	2,800	30,333	29,667	29,000	28,000	7,887	7,887
15: <i>L. ramosus</i>	0,000	30,600	1,000	1,000	2,000	28,258	28,680	28,880	29,000	27,000	28,000	29,000	1,833	1,333	0,000	1,000	2,800	30,333	29,667	29,000	28,000	7,887	7,887
16: <i>L. salinus</i>	1,000	31,600	0,000	2,000	3,000	30,379	30,800	31,000	32,000	28,000	29,000	30,000	2,500	1,000	1,000	0,000	3,000	31,333	30,667	30,000	29,000	8,000	8,000
17: <i>L. salinus</i>	0,000	27,600	1,500	1,500	3,000	26,879	27,300	27,500	28,500	24,500	25,500	26,500	4,000	3,000	2,000	2,000	1,500	21,833	21,167	20,500	19,500	5,500	5,500
18: <i>L. salinus</i>	27,400	1,111	28,400	28,400	24,400	2,537	2,537	2,537	2,537	3,087	3,087	3,087	30,600	30,600	30,600	30,600	28,200	6,433	6,433	7,524	7,524	31,850	32,378
19: <i>L. salinus</i>	29,085	6,987	30,085	30,085	26,085	0,410	0,410	0,410	0,410	3,200	3,200	3,200	30,600	30,600	30,600	30,600	28,200	6,433	6,433	7,524	7,524	31,850	32,378
20: <i>T. elongatum</i>	29,000	9,767	30,000	30,000	26,000	7,941	7,941	7,941	7,941	8,000	8,000	8,000	30,500	30,500	30,500	30,500	28,400	6,887	6,887	8,000	8,000	32,000	32,000
21: <i>T. ponticum</i>	28,000	9,433	29,000	29,000	25,000	6,952	6,952	6,952	6,952	8,000	8,000	8,000	30,500	30,500	30,500	30,500	28,400	6,887	6,887	8,000	8,000	32,000	32,000
22: <i>P. juncea</i> (Boziskiy)	7,000	33,600	8,000	8,000	5,000	30,759	30,287	32,000	34,000	30,000	32,000	32,000	7,500	7,000	7,000	8,000	5,500	30,400	31,154	32,000	31,000	4,000	4,000
23: <i>P. juncea</i> (Mankin)	7,000	33,600	8,000	8,000	5,000	31,258	31,067	33,000	34,000	30,000	32,000	32,000	7,500	7,000	7,000	8,000	5,500	30,400	32,006	32,000	31,000	4,000	4,000

APPENDIX E

CHLOROPLAST DNA K2P AVERAGE NUMBER OF PAIRWISE DIFFERENCES

Appendix E: cpDNA K2P average number of pairwise differences within (diagonal), between (above diagonal) and corrected average pairwise differences between (below diagonal) 19 *Leymus* and four *Triticose* outgroup taxa

OTU species name	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1: <i>L. alpinus</i>	0.0018	0.0219	0.0008	0.0018	0.0008	0.0200	0.0203	0.0205	0.0205	0.0180	0.0197	0.0197	0.0016	0.0011	0.0008	0.0008	0.0008	0.0207	0.0202	0.0158	0.0183	0.0024	0.0033
2: <i>L. ambigua</i>	0.0204	0.0013	0.0211	0.0219	0.0211	0.0053	0.0051	0.0054	0.0024	0.0031	0.0031	0.0047	0.0219	0.0222	0.0211	0.0219	0.0212	0.0030	0.0057	0.0055	0.0055	0.0229	0.0237
3: <i>L. angustus</i>	0.0000	0.0204	0.0000	0.0008	0.0000	0.0191	0.0195	0.0197	0.0197	0.0172	0.0188	0.0188	0.0008	0.0011	0.0000	0.0008	0.0002	0.0198	0.0194	0.0150	0.0180	0.0016	0.0024
4: <i>L. arenarius</i>	0.0008	0.0213	0.0008	0.0000	0.0008	0.0200	0.0204	0.0205	0.0180	0.0197	0.0197	0.0197	0.0016	0.0019	0.0008	0.0016	0.0010	0.0207	0.0202	0.0158	0.0188	0.0024	0.0033
5: <i>L. chinensis</i>	0.0000	0.0204	0.0000	0.0008	0.0000	0.0191	0.0195	0.0197	0.0197	0.0172	0.0188	0.0188	0.0008	0.0011	0.0000	0.0008	0.0002	0.0198	0.0194	0.0150	0.0180	0.0016	0.0024
6: <i>L. chinensis</i>	0.0183	0.0035	0.0183	0.0182	0.0183	0.0017	0.0021	0.0012	0.0047	0.0024	0.0039	0.0040	0.0000	0.0000	0.0000	0.0000	0.0000	0.0047	0.0008	0.0018	0.0048	0.0207	0.0222
7: <i>L. chinensis x tricuspidatus</i>	0.0183	0.0032	0.0183	0.0191	0.0183	0.0001	0.0025	0.0014	0.0048	0.0024	0.0035	0.0038	0.0000	0.0000	0.0000	0.0000	0.0000	0.0047	0.0008	0.0018	0.0048	0.0207	0.0222
8: <i>L. condensatus</i>	0.0187	0.0047	0.0187	0.0205	0.0197	0.0003	0.0002	0.0002	0.0049	0.0024	0.0024	0.0040	0.0000	0.0000	0.0000	0.0000	0.0000	0.0047	0.0008	0.0018	0.0048	0.0207	0.0222
9: <i>L. flavescens</i>	0.0197	0.0018	0.0197	0.0205	0.0197	0.0039	0.0033	0.0049	0.0050	0.0024	0.0024	0.0040	0.0000	0.0000	0.0000	0.0000	0.0000	0.0047	0.0008	0.0018	0.0048	0.0207	0.0222
10: <i>L. linifolius</i>	0.0172	0.0025	0.0172	0.0183	0.0172	0.0016	0.0012	0.0024	0.0024	0.0000	0.0016	0.0016	0.0000	0.0000	0.0000	0.0000	0.0000	0.0047	0.0008	0.0018	0.0048	0.0207	0.0222
11: <i>L. sativus</i> ssp. <i>mojavensis</i>	0.0189	0.0025	0.0189	0.0197	0.0189	0.0031	0.0023	0.0040	0.0024	0.0016	0.0000	0.0032	0.0197	0.0199	0.0188	0.0187	0.0180	0.0026	0.0043	0.0040	0.0040	0.0207	0.0214
12: <i>L. sativus</i>	0.0189	0.0041	0.0189	0.0197	0.0189	0.0032	0.0027	0.0040	0.0040	0.0016	0.0032	0.0000	0.0197	0.0199	0.0188	0.0187	0.0180	0.0026	0.0043	0.0040	0.0040	0.0207	0.0214
13: <i>L. multiflorus</i>	0.0003	0.0207	0.0003	0.0011	0.0003	0.0185	0.0185	0.0200	0.0175	0.0191	0.0191	0.0011	0.0018	0.0008	0.0016	0.0010	0.0010	0.0207	0.0202	0.0158	0.0188	0.0024	0.0033
14: <i>L. racemosus</i>	0.0009	0.0213	0.0008	0.0016	0.0008	0.0191	0.0191	0.0205	0.0205	0.0180	0.0197	0.0197	0.0011	0.0005	0.0011	0.0003	0.0003	0.0209	0.0205	0.0158	0.0188	0.0024	0.0033
15: <i>L. racemosus</i>	0.0000	0.0204	0.0000	0.0008	0.0000	0.0183	0.0183	0.0197	0.0197	0.0172	0.0188	0.0180	0.0000	0.0000	0.0000	0.0000	0.0000	0.0209	0.0205	0.0158	0.0188	0.0024	0.0033
16: <i>L. racemosus</i>	0.0000	0.0213	0.0008	0.0016	0.0008	0.0191	0.0191	0.0205	0.0205	0.0180	0.0197	0.0197	0.0011	0.0005	0.0011	0.0003	0.0003	0.0209	0.0205	0.0158	0.0188	0.0024	0.0033
17: <i>L. sativus</i>	0.0002	0.0204	0.0000	0.0008	0.0000	0.0183	0.0183	0.0197	0.0197	0.0172	0.0188	0.0180	0.0000	0.0000	0.0000	0.0000	0.0000	0.0209	0.0205	0.0158	0.0188	0.0024	0.0033
18: <i>L. sativus</i>	0.0181	0.0009	0.0181	0.0189	0.0181	0.0020	0.0014	0.0029	0.0016	0.0008	0.0008	0.0024	0.0183	0.0188	0.0180	0.0189	0.0180	0.0026	0.0043	0.0040	0.0040	0.0207	0.0214
19: <i>L. sativus</i>	0.0189	0.0045	0.0189	0.0197	0.0189	0.0003	0.0001	0.0003	0.0048	0.0024	0.0040	0.0040	0.0183	0.0188	0.0180	0.0189	0.0180	0.0026	0.0043	0.0040	0.0040	0.0207	0.0214
20: <i>T. elongatum</i>	0.0180	0.0049	0.0180	0.0188	0.0180	0.0040	0.0036	0.0048	0.0048	0.0024	0.0040	0.0040	0.0183	0.0188	0.0180	0.0189	0.0180	0.0026	0.0043	0.0040	0.0040	0.0207	0.0214
21: <i>T. ponticum</i>	0.0180	0.0049	0.0180	0.0188	0.0180	0.0040	0.0036	0.0048	0.0048	0.0024	0.0040	0.0040	0.0183	0.0188	0.0180	0.0189	0.0180	0.0026	0.0043	0.0040	0.0040	0.0207	0.0214
22: <i>P. juncea</i> (Bozashy)	0.0038	0.0223	0.0038	0.0024	0.0016	0.0197	0.0195	0.0207	0.0215	0.0180	0.0207	0.0198	0.0019	0.0024	0.0018	0.0024	0.0015	0.0189	0.0280	0.0196	0.0183	0.0000	0.0000
23: <i>P. juncea</i> (Mankota)	0.0024	0.0230	0.0024	0.0033	0.0024	0.0209	0.0208	0.0222	0.0223	0.0197	0.0214	0.0214	0.0027	0.0033	0.0024	0.0033	0.0024	0.0206	0.0214	0.0205	0.0205	0.0000	0.0000

APPENDIX F

ITS TOTAL AVERAGE NUMBER OF PAIRWISE DIFFERENCES

Appendix F. ITS Total average number of pairwise differences within (diagonal), between (above diagonal) and corrected average pairwise differences between (below diagonal) 19 *Leymus* and two *Triticeae* outgroup taxa *Psathyrostachys juncea* and *Thinopyrum elongatum*

OTU species name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1: <i>L. kalmianensis</i>	4.5000	7.0833	11.8750	4.7500	7.2500	6.7462	6.9833	7.7500	6.0000	7.0000	6.2500	10.3750	9.2500	4.2500	3.2500	3.2500	4.1500	7.3958	7.2262	13.2500	40.5000
2: <i>L. ambigua</i>	4.1061	1.4546	11.1667	5.6667	6.1667	1.6398	1.8778	2.6667	3.4167	5.1667	1.3333	10.1250	9.3333	5.1667	4.1667	4.1667	3.8667	2.3056	2.1111	13.0000	41.4167
3: <i>L. angustus</i>	7.2917	8.1061	4.6667	8.7500	11.0000	10.5265	10.8000	11.5000	10.2500	11.0000	10.5000	12.5000	14.5000	11.0000	10.0000	10.0000	9.7000	11.0625	10.9762	14.0000	38.5000
4: <i>L. arenarius</i>	1.5000	3.9394	5.4167	2.0000	5.5000	5.0265	5.3000	6.0000	4.7500	6.2500	5.0000	8.5000	7.0000	4.5000	3.5000	3.5000	3.2000	5.6667	5.4762	11.5000	40.0000
5: <i>L. chitrensis</i>	5.0000	5.4394	8.6667	4.5000	0.0000	5.5265	5.8000	6.0000	5.2500	7.0000	5.5000	10.0000	9.5000	5.0000	4.0000	4.0000	3.7000	6.1667	5.9762	11.0000	41.0000
6: <i>L. chinensis</i>	4.0043	0.4206	7.7012	3.5346	5.0346	0.9839	1.2793	2.0265	2.7765	4.5266	0.9962	9.4943	8.9749	4.5246	3.5265	3.5265	3.2261	1.6932	1.4789	12.5227	41.1534
7: <i>L. chinensis x trispoides</i>	3.9714	0.3886	7.7048	3.5381	5.0381	0.0254	1.5238	2.2333	3.0500	4.8000	1.2333	9.7333	9.1333	4.8000	3.8000	3.8000	3.5000	1.9667	1.5000	12.9000	41.1333
8: <i>L. condensatus</i>	4.5000	0.9394	8.1667	4.0000	5.0000	0.5346	0.4714	2.0000	3.7500	5.5000	2.0000	10.5000	9.0000	5.5000	4.5000	4.5000	4.2000	2.6667	2.4524	13.5000	42.0000
9: <i>L. flavescens</i>	2.8333	1.7727	7.0000	2.8333	4.3333	1.3679	1.3714	1.8333	1.8333	4.0000	2.7500	9.2500	8.7500	4.2500	3.2500	3.2500	2.9500	3.3750	3.1905	12.2500	40.2500
10: <i>L. limovatus</i>	2.0833	1.7727	8.0000	2.5833	4.3333	1.3670	1.3714	1.8333	0.4167	5.3333	4.5000	7.7500	10.0000	5.0000	4.0000	4.0000	4.3000	5.0208	4.9048	10.5000	41.0000
11: <i>L. salinus</i> ssp. <i>mojavensis</i>	3.5000	0.1061	7.6667	3.5000	5.0000	0.0043	-0.0286	0.5000	1.3333	1.3333	1.0000	9.5000	9.0000	4.5000	3.5000	3.5000	3.2000	1.6667	1.4762	12.5000	41.0000
12: <i>L. molle</i>	6.6250	7.8977	8.6667	6.0000	8.5000	7.5024	7.4714	8.0000	6.8333	3.5833	7.5000	3.0000	12.0000	9.0000	8.0000	8.0000	7.7000	9.9792	9.8810	4.0000	39.0000
13: <i>L. multifidus</i>	6.4643	8.0704	11.6310	5.4643	8.9643	7.9472	7.8357	7.4643	7.2976	6.7976	7.9643	9.9643	1.0714	8.5000	7.5000	7.5000	7.2000	9.4375	9.4286	14.5000	41.8750
14: <i>L. racemosus</i>	1.3333	3.7727	8.0000	2.8333	4.3333	3.3660	3.3714	3.8333	2.6667	1.6667	3.3333	6.8333	7.2976	1.3333	1.0000	1.0000	1.7000	5.1667	4.9762	12.0000	40.0000
15: <i>L. ramosus</i>	1.0000	3.4394	7.6667	2.5000	4.0000	3.0346	3.0381	3.5000	2.3333	1.3333	3.0000	6.5000	6.9643	0.3333	0.0000	0.0000	0.9000	4.1667	3.9762	11.0000	40.0000
16: <i>L. sabulosus</i>	1.0000	3.4394	7.6667	2.5000	4.0000	3.0346	3.0381	3.5000	2.3333	1.3333	3.0000	6.5000	6.9643	0.3333	0.0000	0.0000	0.9000	4.1667	3.9762	11.0000	40.0000
17: <i>L. speciosus</i>	1.3333	2.5727	6.8000	1.6333	3.1333	2.1675	2.1714	2.6333	1.4667	1.0667	2.1333	5.6333	6.0976	0.4667	0.3333	0.3333	1.1333	3.8667	3.6762	10.7000	39.4000
18: <i>L. salinus</i>	4.2367	0.6692	7.8201	3.7576	5.2576	0.2921	0.2957	0.7576	1.5492	1.4451	0.2576	7.5701	7.9927	3.5609	3.2576	3.2576	2.3909	1.8182	2.1310	12.9167	41.2500
19: <i>L. triticoides</i>	4.3345	0.7421	8.0012	3.8345	5.3345	0.3453	0.0964	0.8107	1.6321	1.5964	0.3345	7.7393	8.2512	3.6678	3.3345	3.3345	2.4678	0.5802	1.2834	12.9762	41.2657
20: <i>P. juncea</i>	11.0000	12.2727	11.6667	10.5000	11.0000	12.0308	12.0361	12.5000	11.3333	7.8333	12.0000	2.5000	13.9643	11.3333	11.0000	11.0000	10.1333	12.0076	12.3345	0.0000	38.0000
21: <i>T. elongatum</i>	38.2500	40.6894	36.1667	39.0000	41.0000	40.6615	40.3714	41.0000	39.3333	38.3333	40.5000	37.5000	41.3393	39.3333	40.0000	40.0000	38.8333	40.3409	40.6440	39.0000	0.0000

ITS K2P AVERAGE NUMBER OF PAIRWISE DIFFERENCES

Appendix G ITS K2P average number of pairwise differences within (diagonal), between (above diagonal) and corrected average pairwise differences between (below diagonal) in 19 *Leymus* and two *Trifolium* outgroup taxa *Deschampsiastrum juncea* and *Thinopyrum albatrum*

OTU	species name	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21
1:	<i>L. latrocinensis</i>	0.0695	0.0737	0.1574	0.0650	0.1031	0.0678	0.0679	0.0651	0.0654	0.0589	0.1583	0.1076	0.0556	0.0402	0.0402	0.0540	0.0372	0.0360	0.0360	0.2150	0.8822
2:	<i>L. longus</i>	0.0350	0.0210	0.1449	0.0675	0.0633	0.0237	0.0273	0.0393	0.0276	0.0647	0.0912	0.1312	0.0835	0.0532	0.0364	0.0354	0.0301	0.0335	0.1862	0.4801	
3:	<i>L. angustus</i>	0.1097	0.1150	0.0389	0.1490	0.1490	0.1337	0.1520	0.1337	0.1603	0.1334	0.1324	0.2013	0.1519	0.1324	0.1324	0.1273	0.1432	0.1414	0.2084	0.7528	
4:	<i>L. lanarius</i>	0.0202	0.0324	0.0624	0.0291	0.0951	0.0471	0.0514	0.0627	0.0467	0.0708	0.0460	0.1328	0.0778	0.0689	0.0591	0.0512	0.0468	0.0567	0.0541	0.1857	
5:	<i>L. chilensis</i>	0.0748	0.0558	0.1285	0.0675	0.0400	0.0560	0.0604	0.0538	0.0558	0.0938	0.0549	0.1593	0.1195	0.0762	0.0560	0.0590	0.0846	0.0658	0.0632	0.1778	0.8947
6:	<i>L. chierensis</i>	0.0326	0.0368	0.0681	0.1071	0.0255	0.0489	0.0142	0.1186	0.0300	0.1185	0.0352	0.0144	0.1020	0.0777	0.0420	0.0253	0.0201	0.0246	0.0214	0.1765	0.5653
7:	<i>L. chierus x triflorides</i>	0.0422	0.0665	0.1078	0.0258	0.0493	0.0004	0.0223	0.0331	0.0224	0.0595	0.0179	0.1246	0.0884	0.0762	0.0296	0.0236	0.0243	0.0268	0.0218	0.1822	0.5559
8:	<i>L. condensatus</i>	0.0324	0.0441	0.1181	0.0335	0.0491	0.0081	0.0006	0.0294	0.0327	0.0707	0.0296	0.1389	0.0776	0.0579	0.0406	0.0406	0.0352	0.0396	0.0364	0.1871	0.8820
9:	<i>L. flavescens</i>	0.0299	0.0088	0.0996	0.0259	0.0475	0.0032	0.0031	0.0098	0.0165	0.0518	0.0176	0.1195	0.0817	0.0509	0.0250	0.0250	0.0210	0.0268	0.0242	0.1752	0.8331
10:	<i>L. novatus</i>	0.0164	0.0135	0.0901	0.0245	0.0530	0.0074	0.0046	0.0152	0.0029	0.0815	0.0545	0.1650	0.1134	0.0828	0.0465	0.0466	0.0510	0.0625	0.0608	0.1550	0.4438
11:	<i>L. sinensis ssp. mojavensis</i>	0.0235	0.0015	0.1068	0.0243	0.0478	0.0001	-0.0004	0.0077	0.0001	0.0366	0.0143	0.1196	0.0776	0.0410	0.0242	0.0242	0.0190	0.0242	-0.0214	0.1765	0.9489
12:	<i>L. mollis</i>	0.1079	0.0585	0.1730	0.0561	0.1371	0.0509	0.0614	0.0549	0.0885	0.0847	0.0625	0.1358	0.0159	0.1032	0.0955	0.0855	0.0810	0.0851	0.0851	0.2200	0.7932
13:	<i>L. multiflorus</i>	0.0714	0.0951	0.1739	0.0553	0.0553	0.1115	0.0627	0.0614	0.0549	0.0885	0.0625	0.1358	0.0159	0.1032	0.0955	0.0855	0.0810	0.0851	0.0851	0.2200	0.8811
14:	<i>L. lacunosus</i>	0.0171	0.0320	0.1227	0.0427	0.0655	0.0252	0.0256	0.0335	0.0226	0.0125	0.0424	0.1110	0.0855	0.0193	0.0145	0.0145	0.0249	0.0516	0.0491	0.2003	0.8455
15:	<i>L. laticostus</i>	0.0120	0.0249	0.1130	0.0366	0.0590	0.0182	0.0184	0.0258	0.0168	0.0559	0.0171	0.1024	0.0776	0.0048	0.0000	0.0000	0.0129	0.0347	0.0322	0.1789	0.8440
16:	<i>L. subobtus</i>	0.0120	0.0249	0.1130	0.0366	0.0590	0.0182	0.0184	0.0258	0.0168	0.0559	0.0171	0.1024	0.0776	0.0048	0.0000	0.0000	0.0129	0.0347	0.0322	0.1789	0.8440
17:	<i>L. scalinus</i>	0.0175	0.0113	0.0936	0.0240	0.0464	0.0048	0.0049	0.0122	0.0046	0.0921	0.0036	0.0828	0.0648	0.0070	0.0047	0.0047	0.0185	0.0293	0.0268	0.1744	0.8194
18:	<i>L. scalinus</i>	0.0388	0.0099	0.1105	0.0289	0.0526	0.0043	0.0045	0.0117	0.0054	0.0866	0.0039	0.0629	0.0639	0.0288	0.0215	0.0215	0.0079	0.0264	0.0311	0.1837	0.8604
19:	<i>L. triflorides</i>	0.0395	0.0168	0.1127	0.0303	0.0539	0.0050	0.0014	0.0123	0.0056	0.1108	0.0049	0.0957	0.0683	0.0391	0.0229	0.0229	0.0093	0.0065	0.0166	0.1656	0.8578
20:	<i>P. jumcea</i>	0.1688	0.1701	0.1829	0.1771	0.1778	0.1684	0.1711	0.1824	0.1869	0.1418	0.1684	0.3061	0.2121	0.1907	0.1799	0.1862	0.1706	0.1765	0.1763	0.9000	0.8024
21:	<i>T. elongatum</i>	0.9540	0.9526	0.7333	0.9368	0.8947	0.9491	0.8448	0.8773	0.8249	0.8051	0.8418	0.7710	0.8731	0.8359	0.8440	0.8440	0.8111	0.8472	0.8553	0.8024	0.9000

CHAPTER 3
GENETIC ANALYSIS OF *LEPIDIUM* (BRASSICACEAE)
IN WESTERN NORTH AMERICA¹

ABSTRACT

The genus *Lepidium* is a member of the mustard family (Brassicaceae). Fifty-eight accessions representing five *Lepidium* species and four varieties were collected throughout regions of western North America. The level of genetic variation among *Lepidium* was tested using barcoding sequences from the *trnH-psbA*, *trnL-trnL-trnF* regions of the chloroplast genome, and the 18S-5.8S-26S nuclear ribosomal DNA internal transcribed spacer regions. One mutation distinguished all *L. papilliferum* from all but three *L. montanum* accessions. Significant levels of genetic differentiation were found for cpDNA ($F_{st} = 0.11660$) and for the internal transcribed spacer ($F_{st} = 0.33778$) between *L. montanum* and *L. papilliferum* based on the Kimura's 2-parameter test of sequence divergence. Divergence time estimates between *L. montanum* and *L. papilliferum* range from 22,400 to 10,400 years ago, based on a nucleotide sequence divergence (0.008%) for the chloroplast DNA sequences and 136,000 to 74,000 years ago based on a nucleotide sequence divergence (0.124%) for internal transcribed spacer sequences. The recent divergence times suggest a very close relationship between slickspot peppergrass, *L. papilliferum* and mountain pepperweed, *L. montanum*. Additional sampling with less geographical bias, may reveal more continuous relationships between *L. montanum* and *L. papilliferum*.

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INTRODUCTION

Lepidium includes 75 species worldwide, of which 38 both introduced and native, are found in North America. Several species and varieties of pepperweed have been subject to frequent changes in classification. It has long been suggested by several taxonomic authors that the North American *Lepidium* complex needs further examination in order to reach an agreement on the taxonomy of its species (Holmgren et al., 2005). Particular interest has developed in the relationship between *L. montanum* and *L. papilliferum*. When the U.S. Fish and Wildlife Service proposed to list *L. papilliferum* as an endangered species, the question was raised as to whether *L. papilliferum* merits species status or if it should be considered the same species or a subspecies of *L. montanum*. According to Section 3 (16), the Endangered Species Act (ESA) of 1973 the term “species” to include “any subspecies of fish or wildlife or plants, and any distinct population segment of any species or vertebrate of fish or wildlife, which interbreeds when mature.”

Although first described as *L. montanum* var. *papilliferum* (Henderson, 1900), *L. papilliferum* was later classified as a distinct species based on its unique growth habit, short lifespan, and unusual pubescence (Nelson and McBride, 1913; Rollins, 1993). Recent studies declare that *L. papilliferum* warrants species recognition based on differences in morphology, as well as life history traits including seed dormancy and seed bank characteristics (Meyer and Allen, 2005).

Prior analyses have focused on resolving the continental origins, as well as migration and divergence time in *Lepidium* worldwide. Molecular studies have utilized

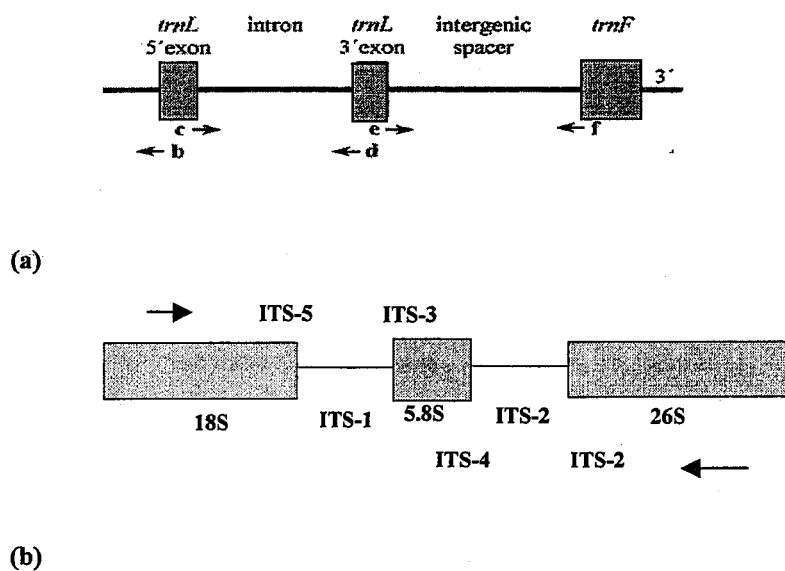


Figure 3-1 (a) Diagram of the cpDNA *trnL* intron and the *trnL-trnF* intergenic spacer region primers c through f (Taberlet et al., 1991). Shaded boxes represent exons and are not drawn to scale; the intron and spacer region are shown by solid lines and are drawn approximately to scale. (b) internal transcribed spacer 18S-5.8S-26S.

nuclear rDNA internal transcribed spacer (ITS) as well as non-coding cpDNA sequences, and single-copy nuclear DNA sequences (PISTILLATA) to clarify the relationships among some *Lepidium* taxa (Lee et al., 2002). Adequate information to resolve species relationship has been attained in some taxa using chloroplast DNA (cpDNA). Chloroplast DNA has also shown promise as a source of DNA barcoding sequences (Kress et al., 2005). Universal PCR primers flanking noncoding sequences of the cpDNA, *trnL-trnL-trnF* region (Taberlet et al., 1991) rank among the first and most widely used regions in plant molecular systematics (Figure 3-1(a)) (Shaw et al., 2005). The *trnL-trnL-trnF* region has been used to discriminate species and resolve phylogenetic relationships among many *Lepidium* taxa of the world (Mummenhoff et al., 1995, 2001, 2004).

The internal transcribed spacer (ITS) region of the 18S-5.8S-26S nuclear ribosomal genes has become one of single most commonly used markers for phylogenetic studies at the species level in plants (Figure 3-1(b)) (Álvarez and Wendel, 2003; Baldwin et al., 1995), and also shows some promise as a plant DNA barcoding locus at the species level (Kress et al., 2005). Properties of the ITS locus claimed to be advantageous include biparental inheritance, universality, simplicity, intragenomic uniformity that in many cases may eliminate confounding variation within plants leaving only species- and clade-specific character-state changes, and low functional constraint allowing neutral evolution. Another advantage of the ITS region is that it can be amplified in two smaller fragments (ITS1 and ITS2), using the 5.8S as a universal primer bridge, which has proven especially useful in degraded samples (Kress et al., 2005). The ITS1 and ITS2 regions have been used to construct a phylogeny of diverse *Lepidium* species from many regions of the World, including one reference specimen of *L. montanum* (Bowman et al., 1999; Mummenhoff et al., 2004).

A molecular analysis was conducted to measure sequence divergence and test the hypotheses that unique polymorphisms would distinguish *L. montanum* and *L. papilliferum*. Previously described polymerase chain reaction (PCR) primers flanking the cpDNA *trnL-trnL-trnF* region and the ITS1 and ITS2 regions were used to sequence *L. papilliferum*, as well as five varieties of *L. montanum*, (*L. montanum* var. *alpinum*, *L. montanum* var. *montanum*, *L. montanum* var. *jonesii*, *L. montanum* var. *wyomingense*, and *L. montanum* var. *alyssoides*). Other *Lepidium* included for reference were: *L. fremontii*, *L. lasiocarpum*, *L. oblongum*, *L. virginicum*, *L. perfoliatum*, and *L. draba*.

LITERATURE REVIEW

Traditionally, *Lepidium* has been classified based on similarities in reproductive morphology and geographic distribution (Thellung, 1906; Hewson, 1981). The relationship of the three main lineages of the *Lepidium* complex *Monoploca*, *Lepia*, and *Lepidium*, have been characterized based on ancestral character state reconstruction of 19 *Lepidium* species (Lee et al., 2002; Bowman et al., 1999; Mummenhoff et al., 2001). The number of stamens in the floral structure is conserved in most Brassicaceae taxa, but is often reduced in *Lepidium*. These similarities could have arisen from convergent evolution, yet previous cpDNA, ITS, and PI intron analyses suggest Australian, North and South American *Lepidium* more likely resulted from introgression of morphological traits via allopolyploid hybridization (Lee et al., 2002). Ten of 13 species from North and South America are thought to have allopolyploid origins based on 1) phylogenies with multiple sequences of the same species clustered in separate clades and 2) the lack of lateral stamens in suspected allopolyploids. A 0-2.2% divergence rate indicates the radiation of *Lepidium* into multiple continents including North and South America, South Africa, and Australia most likely occurred no more than 2.2 mya, corresponding with the changing climate and increased bird migration in the Pliocene/Pleistocene geological periods. A lack of genetic differentiation among *Lepidium* taxa, and the similar divergence time estimates from Asia to North America, compared to North and South America may indicate a rapid radiation (Mummenhoff et al., 2001, 2004).

Prior analyses have solidified the close relationship of *L. montanum* and *L. fremontii* among other North American taxa including *L. lasiocarpum*, *L. densiflorum*,

and *L. oblongum*. Still, the relationship between several western North American species with similar reproductive morphology and proximal geographic distribution remains unexplained. Varieties of *L. montanum* ((Nutt.) Torr. and A.Gray, 1838), have been subject to frequent changes in classification. Several taxonomic authors suggest that further examination of *L. papilliferum* ((L.F. Hend) A. Nelson and J.F. Macbr., 1913), *L. montanum* var. *montanum* ((Nutt.) Torr. and A.Gray, 1838), *L. montanum* var. *jonesii* ((Rydb.) C.L. Hitchcock, 1936; 1950), *L. montanum* var. *alyssoides* ((A. Gray) M. E. Jones, 1893), and *L. eastwoodiae* (Wooton, 1898), is necessary to clarify the relationship among these taxa. Populations of *Lepidium montanum* are found in a diversity of habitats in montane areas between 1200-2100 (2700) m, in the Intermountain and western Rocky Mountain regions of North America. *Lepidium montanum* Nutt. was first published in *A Flora of North America* (1838). The type information provided by Thomas Nuttall (1786-1859) states: "Plains of the Rocky Mountains, on the western side, to the borders of the Oregon."

Lepidium papilliferum typically occurs in flat to gently sloping terrain in the "slickspot" interspaces of sagebrush (*Artemisia* sp.) steppe habitats between 670 –1650 m in the volcanic plains of the Snake River Plain and the Owyhee Plateau, in southwest Idaho. It is assumed to be endemic to this region only. *Lepidium papilliferum* has been reported to occur within, at the edge, or outside of slickspots, and it can often be found on top of animal burrows and occasionally along roadsides. Slickspots are classified by their unique soil and water retention characteristics. These soils have very thin silt and restrictive hardpan layers above a very deep moist clay layer. The soils adjacent to slickspots have a much more profound surface silt layer with a moist clay layer found

immediately below (Fisher et al., 1996; Meyer and Allen, 2005).

The vast diversity of soil habitats in which *L. montanum* varieties are found have not been assessed with equal scrutiny. *Lepidium montanum* var. *montanum* is also found in highly alkaline areas, in saline flats among *Sarcobatus*, *Atriplex*, and *Distichlis* across central and southern Idaho. These habitats are similar and in proximity to locations where *L. papilliferum* resides. Yet *L. montanum* var. *montanum* differs from *L. papilliferum* in that it is also found in sandy washes and deserts, clayey hillsides, gravel slopes, and in the crevices of outcroppings. Its distribution covers a much larger area, from western Montana west to eastern Oregon, Nevada, and California and south through central and western Utah to Arizona. *Lepidium montanum* var. *jonesii* has been documented frequently from eastern and southern Utah and more seldomly in northern Arizona, New Mexico, western Colorado and southeastern Nevada (NYBG website). *Lepidium montanum* var. *jonesii*, consists of generally long-lived perennials found in fine-grained clay-silt (gumbo) and limestone gravel soil substrates, in a wide range of habitats including sand washes in deserts, bases of sandstone cliffs, rocky hillsides and pinyon-juniper woodlands (Rollins, 1993; Holmgren et al., 2005). *L. montanum* var. *alyssoides* is often recognized as a separate species *L. alyssoides* (Kuntze, 1891; Rollins, 1993; Holmgren et al., 2005). Authors differ as to the distribution *L. montanum* var. *alyssoides*. The Intermountain Flora (Holmgren et al. 2005) treatment limits *L. montanum* var. *alyssoides* communities to west Texas, New Mexico, southwest Colorado, and sparsely through Arizona, southern Nevada, and southern Utah. The Flora of Wyoming (Dorn, 2001) and Cruciferae of Continental North America (Rollins, 1993) expand this distribution to include southeastern Idaho and Wyoming. *Lepidium montanum* var.

wyomingense C. L. Hitchcock, is found in southeastern Idaho, throughout southern Wyoming, and Colorado. It is also suspected to occur in northeastern Utah (N. Holmgren, personal communication).

Lepidium montanum are seldom annual, sometimes biennial, mostly perennial plants 0.4-4 (5.5) dm tall. The stems are few to many, surmounting a crown or short-branched caudex above or below the ground. The stems are simple to freely branching, giving a globular shape to the plant. The herbage is glabrous to densely pubescent. All *L. montanum* seldom deviate from their floral plan of four oblong to broadly obovate sepals (1-2 mm), four white to pale-cream petals (2-3.5 (4.5) mm), six stamens, and two united carpels. *Lepidium* is derived from the Greek name *Lepis* or lepidion that refers to the scale-like appearance of its silicles. The silicles of *L. montanum* are ovate to suborbicular, 2.1-4.4 mm long, 1.7-3.5 (4) mm wide, slightly winged with a slight notch at the apex of the glabrous or sparsely pubescent fruit. The inflorescence is a many-flowered raceme 1-2 cm long congested in flower then elongating in fruit. The pedicels subtending the fruit are (2.8) 3.5-8.5 mm long arched and spreading. The rachis and pedicels are puberulent or glabrous (Rollins, 1993; Holmgren et al., 2005). The basal leaves, a qualifying character of *Lepidium*, are often early deciduous occurring simultaneously or before the flowers and fruit.

Despite its many overlapping characteristics, *L. papilliferum* has been recognized as a separate species based on the restricted and exclusive distribution of *L. papilliferum* relative to *L. montanum*, several differing morphological characters, and the specific “slickspot” habitat in which *L. papilliferum* grows. According to Rollins (1993) and Holmgren et al. (2005), physical characteristics found only in *L. papilliferum* include 1)

clavate-shaped hairs found on the leaves and stems as well as on the filaments of the stamens, 2) the pinnate division of all leaves on a plant (as opposed to some being entire as found in *L. montanum*), and 3) the silicles of are broadly ovate to nearly orbicular, they do not taper to the apices (unlike the tapered, ovate shape of *L. montanum* silicles), and they are without even vestiges of wings toward the apices. Despite these differences, other floras continued to recognize slickspot pepperweed as *L. montanum* var.

papilliferum (Hitchcock et al., 1964; Davis, 1952; Hitchcock and Cronquist, 1973).

Lepidium montanum var. *jonesii* are typically long-lived shrub-like plants, usually between 1.5-3.5 dm tall, the stems arising from a branched caudex or short-branched woody base. The basal leaves have three to five deep lobes, while the cauline leaves are generally entire to shallowly lobed. *Lepidium montanum* var. *jonesii* flowering time ranges from April to July (Rollins, 1993; Holmgren et al., 2005).

Lepidium montanum var. *alyssoides* is distinguished from other taxa by its woody, shrub-like appearance and by its entire, linear (< 2mm wide) cauline leaves. The petals (2-3 mm) and fruit (1.8-2.8 mm wide) are somewhat shorter and narrower than other *L. montanum* taxa. Flowering time ranges from May to September (Rollins, 1993; Holmgren et al., 2005). *L. eastwoodiae* (not included in this study) is classified as annual, biennial, or perennial, with a wide range of height, 4-15 dm. July-September flowering times vary from other *Lepidium*.

MATERIALS AND METHODS

Fifty-eight accessions including five *Lepidium* species and four varieties were collected throughout several states in western North America (Table 3-1, Figure 3-2).

To insure collections include as much of the distributional range of *L. montanum* as possible, research of herbaria records from several institutions was necessary to pinpoint the potential localities of the species of interest. Research entailed visits to herbaria as not all have current online database catalogs of their collections available. All *L. papilliferum* collections were coordinated through Tom Perry in the Idaho Governor's Office of Species Conservation (OCS) and Howard Hedrick and Bill Baker in the Idaho BLM Jarbidge Field Office.

The time frame in which plant collections were completed was limited to anthesis to ensure the identity of the specimens and to obtain the best quality DNA possible. When collecting, specific site information was recorded including coordinates, elevation, habitat information, and directions to the site.

Lepidium papilliferum collections included photos for each site, as well as a detailed synopsis about the plants' proximity to the nearest slickspot. These data were collected to establish whether *L. papilliferum* populations conformed to the slickspot-specific habitat, one of the four qualifying characters that distinguish *L. papilliferum* as a species. At least one representative specimen was collected and pressed on site. For DNA extractions, cuttings were collected from the freshest youngest leaves possible, which were collected and stored in silica-drying agent to preserve the DNA at the highest quality possible. The specimens were identified in the field and verified by *Lepidium* specialist, N. H. Holmgren, in the laboratory.

Twenty-four *L. papilliferum* and two *L. montanum* var. *montanum* were collected by Robert N. Schweigert, 29 *L. montanum* varieties were collected by C.M. Culumber, and additional *Lepidium* specimens were located by Bonnie Heidel, Tom Jones, and

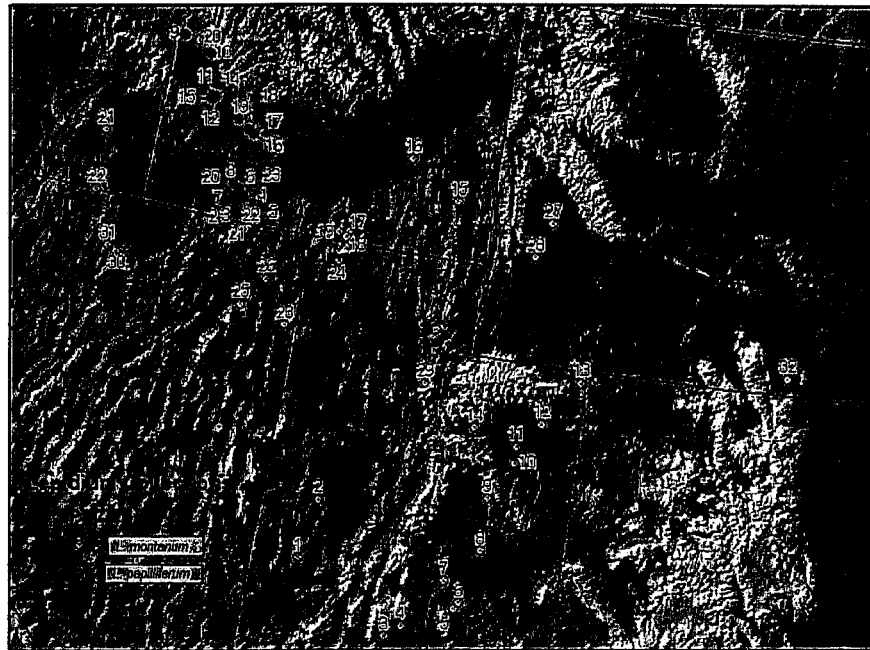


Figure 3-2 Distribution map of *Lepidium* accessions included in the study. Green points represent *Lepidium montanum* (LEMO) accessions, purple points *Lepidium papilliferum* (LEPA), numbers correspond to the sequence ID column in Table 1.

Steve Larson. Specimens were deposited at the Intermountain Herbarium, Utah State University, Logan, Utah. Each specimen or group of specimens from the same locality were assigned a Universal Taxonomic Code (UTC), as well as a sample ID, to ease sample recognition in the analyses. *Lepidium montanum* sample IDs begin with LEMO, and *L. papilliferum* with LEPA, and were followed by a distinctive number representing the collection site, and an alphabetical character for each duplicate specimen from collections sites (Table 3-1). Information for each accession was entered into the Intermountain Herbarium database. Voucher information can be viewed on the Global Biodiversity Information Facility (GBIF) website <http://www.gbif.org/>.

DNA Isolation and Barcoding- DNA was isolated from dry leaves using DNeasy 96 plant DNA extraction kit (four 96-well plates) (Quiagen, Valencia, California, USA). The quantity and quality of DNA was determined by agarose gel electrophoresis.

A subset of 96 DNA samples were selected for DNA sequencing, including at least one plant (usually two) from each of the 21 LEPA and 32 LEMO collection sites. The chloroplast *trnL* intron was PCR amplified using the *trnL* c and d primers described by Taberlet et al. (1991). Likewise, the chloroplast *trnL-F* intergenic spacer region was amplified using the *trnL* e and *trnF* f primers. The ITS1, 5.8S, and ITS2 region was PCR amplified using the ITS-5a (Stanford et al., 2000) and ITS-4 (White et al., 1990) primers.

Amplifications of DNA sequences were performed in 50- μ l volumes of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, and 0.1% Triton X-100, 2 mM MgCl₂, 0.4 μ M primers, 1 unit *Taq* DNAP, and approximately 30 ng of plant DNA using the following temperature profile: 94° C (1 minute); 35 cycles of 94° C denaturing (30 seconds), 55° C annealing (45 seconds), and 72° C extension (2 minute); 72° C extension (5 minute). The PCR amplification products were purified using the QuickStep 2 96-Well PCR Purification Kit (Edge BioSystems, Gaithersburg, Maryland). Bidirectional sequencing reactions were performed using 0.5 μ l of BigDye Terminator v3.1 Cycle Sequencing RR-100 reagent, 2 μ l of BigDye Terminator v3.1 5X Sequencing Buffer, 1 μ l of 2 μ M primer, and 0.5 μ l of purified PCR product in a 10- μ l reaction volume as recommended by the manufacturer (Applied Biosystems, Foster City, California), with the same primers used for PCR amplification. Products from the sequencing reactions were purified using the Performa DTR V3 96-Well Short Plate Kit (Edge BioSystems, Gaithersburg, Maryland). Aqueous eluates were fractionated on an ABI3730 capillary electrophoresis

Table 3-1 List of *Lepidium* accessions included in study. UTC numbers were assigned at the Intermountain herbarium, Utah State University, Logan, Utah.

UTC #	SEQ ID	Species	Variety	County	State	Latitude	Longitude	Elevation	Date
246228	LEMO_1	<i>L. montanum</i>	montanum	Beaver	UT	38.4544	113.2739	6636	5/17/2006
246230	LEMO_2	<i>L. montanum</i>	montanum	Millard	UT	39.1262	113.1080	4620	5/17/2006
246231	LEMO_3	<i>L. montanum</i>	jonesii	Garfield	UT	37.7485	111.7064	6306	5/26/2006
246232	LEMO_4	<i>L. montanum</i>	jonesii	Garfield	UT	37.8958	111.4469	6476	5/28/2006
246233	LEMO_5	<i>L. montanum</i>	jonesii	Wayne	UT	38.1732	110.6659	4998	5/29/2006
246234	LEMO_6	<i>L. montanum</i>	jonesii	Wayne	UT	38.2473	110.6052	4728	5/29/2006
246235	LEMO_7	<i>L. montanum</i>	jonesii	Wayne	UT	38.4646	110.8927	4762	5/29/2006
246236	LEMO_8	<i>L. montanum</i>	jonesii	Emery	UT	38.8637	110.3608	4278	5/29/2006
246237	LEMO_9	<i>L. montanum</i>	jonesii	Emery	UT	39.4524	110.3715	6095	5/29/2006
246238	LEMO_10	<i>L. montanum</i>	jonesii	Duchesne	UT	39.8795	110.0330	6516	5/30/2006
246239	LEMO_11	<i>L. montanum</i>	jonesii	Duchesne	UT	40.0658	110.0204	5140	5/30/2006
246240	LEMO_12	<i>L. montanum</i>	jonesii	Uintah	UT	40.3519	109.6749	5281	5/30/2006
246241	LEMO_13	<i>L. montanum</i>	jonesii	Daggett	UT	40.8681	109.1145	5688	5/31/2006
246242	LEMO_14	<i>L. montanum</i>	jonesii	Duchesne	UT	40.2010	110.7757	6280	6/1/2006
246243	LEMO_15	<i>L. montanum</i>	wyominense	Caribou	ID	42.6578	111.6532	5736	6/5/2006
246244	LEMO_16	<i>L. montanum</i>	montanum	Bingham	ID	43.0400	112.5425	4403	6/6/2006
246245	LEMO_17	<i>L. montanum</i>	montanum	Cassia	ID	42.1218	113.4094	5138	6/7/2006
246246	LEMO_18	<i>L. montanum</i>	montanum	Cassia	ID	42.0553	113.4326	5127	6/7/2006
246247	LEMO_19	<i>L. montanum</i>	montanum	Cassia	ID	42.0909	113.5701	5168	6/7/2006
246248	LEMO_20	<i>L. montanum</i>	montanum	Gem	ID	43.8756	116.6318	2236	6/7/2006
246249	LEMO_21	<i>L. montanum</i>	montanum	Malheur	OR	42.6755	117.8649	3840	6/8/2006
246250	LEMO_22	<i>L. montanum</i>	montanum	Malheur	OR	42.0097	117.7981	4440	6/8/2006
246251	LEMO_23	<i>L. montanum</i>	montanum	Elko	NV	40.9667	114.1898	4580	6/8/2006
246252	LEMO_24	<i>L. montanum</i>	montanum	Box Elder	UT	41.7814	113.4860	5314	6/9/2006
246258	LEPE_1	<i>L. perfoliatum</i>	-	Owyhee	ID	42.3039	115.3706	5068	5/24/2006
246259	LEPA_2	<i>L. papilliferum</i>	-	Owyhee	ID	42.2384	115.4259	5080	5/24/2006
246260	LEPA_3	<i>L. papilliferum</i>	-	Owyhee	ID	42.2029	115.4187	5318	5/25/2006
246261	LEPA_4	<i>L. papilliferum</i>	-	Owyhee	ID	42.2744	115.2935	5134	5/25/2006
246262	LEPA_5	<i>L. papilliferum</i>	-	Owyhee	ID	42.2716	115.2971	5153	5/25/2006
246263	LEPA_6	<i>L. papilliferum</i>	-	Owyhee	ID	42.3039	115.3706	5068	5/26/2006
246264	LEPA_7	<i>L. papilliferum</i>	-	Owyhee	ID	42.2469	115.4565	5039	5/26/2006
246265	LEPA_8	<i>L. papilliferum</i>	-	Owyhee	ID	42.3539	115.3930	5001	5/26/2006
246266	LEPA_9	<i>L. papilliferum</i>	-	Payette	ID	43.8966	116.8720	2493	5/28/2006
246267	LEPA_10	<i>L. papilliferum</i>	-	Ada	ID	43.7533	116.3586	2849	5/29/2006
246268	LEPA_11	<i>L. papilliferum</i>	-	Ada	ID	43.2996	116.1759	3228	5/30/2006
246269	LEPA_12	<i>L. papilliferum</i>	-	Ada	ID	43.2972	116.1175	3257	5/30/2006
246270	LEPE_13	<i>L. perfoliatum</i>	-	Ada	ID	43.2987	116.1101	3246	5/30/2006
246271	LEPA_14	<i>L. papilliferum</i>	-	Ada	ID	43.3002	116.1099	3252	5/30/2006
246272	LEPA_15	<i>L. papilliferum</i>	-	Ada	ID	43.2747	116.2082	3302	5/31/2006
246273	LEPA_16	<i>L. papilliferum</i>	-	Elmore	ID	42.9816	115.3447	3025	5/31/2006
246274	LEPA_17	<i>L. papilliferum</i>	-	Owyhee	ID	43.0111	115.4252	3135	5/31/2006
246275	LEPA_18	<i>L. papilliferum</i>	-	Owyhee	ID	43.0240	115.4400	3162	6/1/2006
246276	LEPA_19	<i>L. papilliferum</i>	-	Owyhee	ID	43.1078	115.6365	3231	6/1/2006
246277	LEPA_20	<i>L. papilliferum</i>	-	Owyhee	ID	42.2777	115.4251	4998	6/3/2006
246278	LEPA_21	<i>L. papilliferum</i>	-	Owyhee	ID	42.2496	115.4080	5134	6/3/2006
246279	LEPA_22	<i>L. papilliferum</i>	-	Owyhee	ID	42.2595	115.3052	5194	6/3/2006
246280	LEPA_23	<i>L. papilliferum</i>	-	Owyhee	ID	42.2835	115.3415	5314	6/4/2006
246253	LEMO_25	<i>L. montanum</i>	montanum	Elko	NV	41.1026	114.9987	5905	6/19/2006
246254	LEMO_26	<i>L. montanum</i>	montanum	Elko	NV	41.4294	114.6527	5500	6/20/2006
246255	LEMO_27	<i>L. montanum</i>	alyssooides	Sublette	WY	42.5711	109.9664	6890	6/29/2006
246284	LEMO_28	<i>L. montanum</i>	alyssooides	Lincoln	WY	42.1768	110.1872	6549	6/30/2006
246255	LEMO_29	<i>L. montanum</i>	alpinum	Salt Lake	UT	40.6038	111.6901	8993	7/17/2006
246256	LEMO_30	<i>L. montanum</i>	montanum	Humboldt	NV	41.3874	117.2448	4550	6/21/2006
246257	LEMO_31	<i>L. montanum</i>	montanum	Humboldt	NV	41.4259	117.3996	4481	6/21/2006
246283	LEMO_32	<i>L. montanum</i>	montanum	Albany	WY	41.1817	105.7233	7205	6/16/2006
246281	LELA	<i>L. lasiocarpum</i>	-	Mineral	NV	38.5875	118.1744	5213	6/14/2006
246282	LEDR	<i>L. draba</i>	-	Utah	UT	40.0575	-111.5620	4600	5/28/2006

instrument by the Utah State University Center for Integrated Biosystems.

Sequencher 4.6 (Gene Codes Corp, Ann Arbor, Michigan) was used to visually inspect all contigs and resolve all discrepancies between complementary sequencing strands. Ambiguous base calls were only used if both strands showed evidence of the same, roughly equal mixed base composition.

Genetic analysis of chloroplast and ITS sequence variation- Phenetic similarities among *L. montanum*, *L. papilliferum*, and all other available *Lepidium* sequences including over 50 other taxa available in GenBank (Mummenhoff et al., 1995, 2001, 2004; Bowman et al., 1999) were compared by UPGMA analysis based on the total number of character differences in PAUP (version 4.0b10; Swofford, 2000). Gaps were treated as missing data in PAUP, but gapcodes were included in the total number of character differences. This provided a simple and deterministic genetic comparison of all available sequences, which is somewhat independent of the parsimony-based phylogenetic searches described below.

Parsimony analysis of all *L. montanum* sequences, all *L. papilliferum* sequences, and selected reference taxa assumed unordered and unweighted character states using a heuristic search in PAUP (version 4.0b10; Swofford, 2000) with TBR branch swapping, starting with a simple stepwise addition procedure. Random sequence addition procedures were tested using 100 replications. All characters were considered unordered and equal weighted. Bootstrap support values were obtained from 500 replicates using a heuristic search and simple addition. A 60 (sec) time limit per rep was used for bootstrap analysis of the chloroplast DNA analysis, which was more than enough time to complete a full-dataset heuristic search with simple addition. The elimination of all but two ITS

reference sequences (outgroups) was used to simplify heuristic searches of all available *L. montanum* and *L. papilliferum* sequences without the complexity of considering all other available *Lepidium* sequences, which have already been analyzed (Mummenhoff et al., 1995, 2001, 2004; Bowman et al., 1999).

Analysis of molecular variance (AMOVA) based on Euclidean distances was computed using Arlequin (Excoffier et al., 1992). Kimura's 2-parameter (K2P) and total average number of differences among haplotypes, was used to calculate the number of differences between (PiXY), the average number of pairwise differences within (PiX and PiY), and the corrected average pairwise differences among *L. montanum* and *L. papilliferum* ($PiXY - (PiX + PiY) / 2$). Kimura's 2-parameter sequence divergences were used to estimate the time of divergence between *L. montanum* and *L. papilliferum*. Kimura two-parameter distance measures were converted into a simple molecular clock using $K=2Tk$, T is the divergence time, k is the constant rate of nucleotide substitution (Kimura, 1981), and K is the corrected total number of base substitutions per site that separate two sequences. We assumed the divergence time between *L. montanum* and *L. papilliferum* would correspond to the mean divergence time estimates between Australian and New Zealand *Lepidium*, based on mean K2P divergence in the *trnT/L* spacer and the *trnL* intron, as well as the ITS region (Mummenhoff et al., 2004). The molecular clock by which Mummenhoff et al. (2004), based his estimates was an assumed divergence time of $2.5-5 \times 10^6$ (million) years ago (Mya). This was based on fossil data of *Rorippa* (Brassicaceae) (Mai, 1995) and a minimum K2P sequence divergence rate of 1.8% (*trnT/L* spacer, *trnL* intron) and 4.4% (ITS) between closely related *Rorippa* and *Cardamine* (Franzke et al., 1998).

RESULTS

ITS DNA sequences- Relative to other taxa of North American and from other continents, a high degree of identity was detected among all *L. montanum*, *L. papilliferum*, and *L. fremontii* sequences, including those from GenBank (Mummenhoff et al., 2001) evidenced by the UPGMA analysis of the nuclear ribosomal DNA ITS region (Figure 3-3). *Lepidium montanum* and *L. papilliferum* ITS amplicons ranged from 731 to 748 bp long, most being 748 bp long, with 42 bp of primer sequence included in these lengths. The alignment of *L. montanum* and *L. papilliferum* ITS sequences was 723 bp, with 25 bp of primer sequence removed. There were a total of 163 operational taxonomic units (OTUs), including multiple haplotypes within 13 OTUs, in the ITS phylogenies. Ambiguous base calls, with roughly equal mixed base signals on both sequencing strands, were observed in LEMO-01A, LEMO-01E, LEMO-12A, LEMO-12C, LEMO-13B, LEMO-14D, LEMO-15A, LEMO-15C, LEMO-16B, LEMO-24A, LEMO-27B, LEMO-28C, and LEMO-29B. These ambiguous base calls were resolved into two haplotypes for each of these 13 OTUs. These haplotypes differed by only one ambiguous base for seven OTUs (LEMO-1A, LEMO-1E, LEMO-13B, LEMO-14D, LEMO-15A, LEMO-15C, and LEMO-24A), two ambiguous bases for three OTUs (LEMO-12A, LEMO-16B, and LEMO-29B), three ambiguous bases in one OTU (LEMO-12C), and five ambiguous bases in two OTUs (LEMO-27B and LEMO28C).

The overall alignment of LEMO, LEPA, and all other reference taxa, including 55 OTUs created from 110 GenBank ITS1 and ITS2 sequences (Mummenhoff et al., 2001), was 742 bp. Another 48 gap codes were included to account for relatively small indels.

Figure 3-3 UPGMA distance analysis among *Lepidium montanum* (LEMO), *L. papilliferum* (LEPA), and other North American *Lepidium* taxa based on the total number of differences (substitutions or indels) among nuclear ribosomal DNA internal transcribed spacer (ITS) haplotypes, with bootstrap support values determined from 1000 sample reps.

The ITS1 and ITS2 GenBank sequences (Mummenhoff et al., 2001) were concatenated for each OTU, after confirming that they belonged to the same plant genotype.

Heuristic searches, including all available *Lepidium* sequences, or even just all available North American taxa, found numerous equally parsimonious trees. As shown in Figure 3-4, *L. montanum*, *L. papilliferum*, and *L. fremontii* ITS sequences showed a very high degree of identity to each other relative to other taxa. *Lepidium perfoliatum* sequences were not available in GenBank and our *L. lasiocarpum* ITS PCR failed. Thus we limited the reference taxa used in our final heuristic searches to the North American *L. lasiocarpum* and *L. virginicum* taxa, which have ITS sequences most like *L. montanum*, *L. papilliferum*, and *L. fremontii*. The ITS sequences of these five taxa included 10 parsimony-uninformative variable characters and 37 parsimony-informative variable characters.

Within *L. montanum*, *L. papilliferum*, and *L. fremontii*, three North American taxa, there were eight parsimony-uninformative characters and 11 parsimony-informative characters. A heuristic parsimony search of this dataset found one tree with 19 steps and no homoplasy among the *L. montanum*, *L. fremontii*, and *L. papilliferum* taxa. Figure 3-4 shows the single most parsimonious tree obtained using simple heuristic searches or a random sequence addition heuristic search with 1000 replicates. Five parsimony-uninformative characters were unique to the *L. montanum* sequence in GenBank (Mummenhoff et al., 2001). If the latter sequence is deleted, there are only three parsimony-uninformative and 11 parsimony-informative characters. A heuristic parsimony search of this dataset found one tree with 14 steps and no homoplasy among

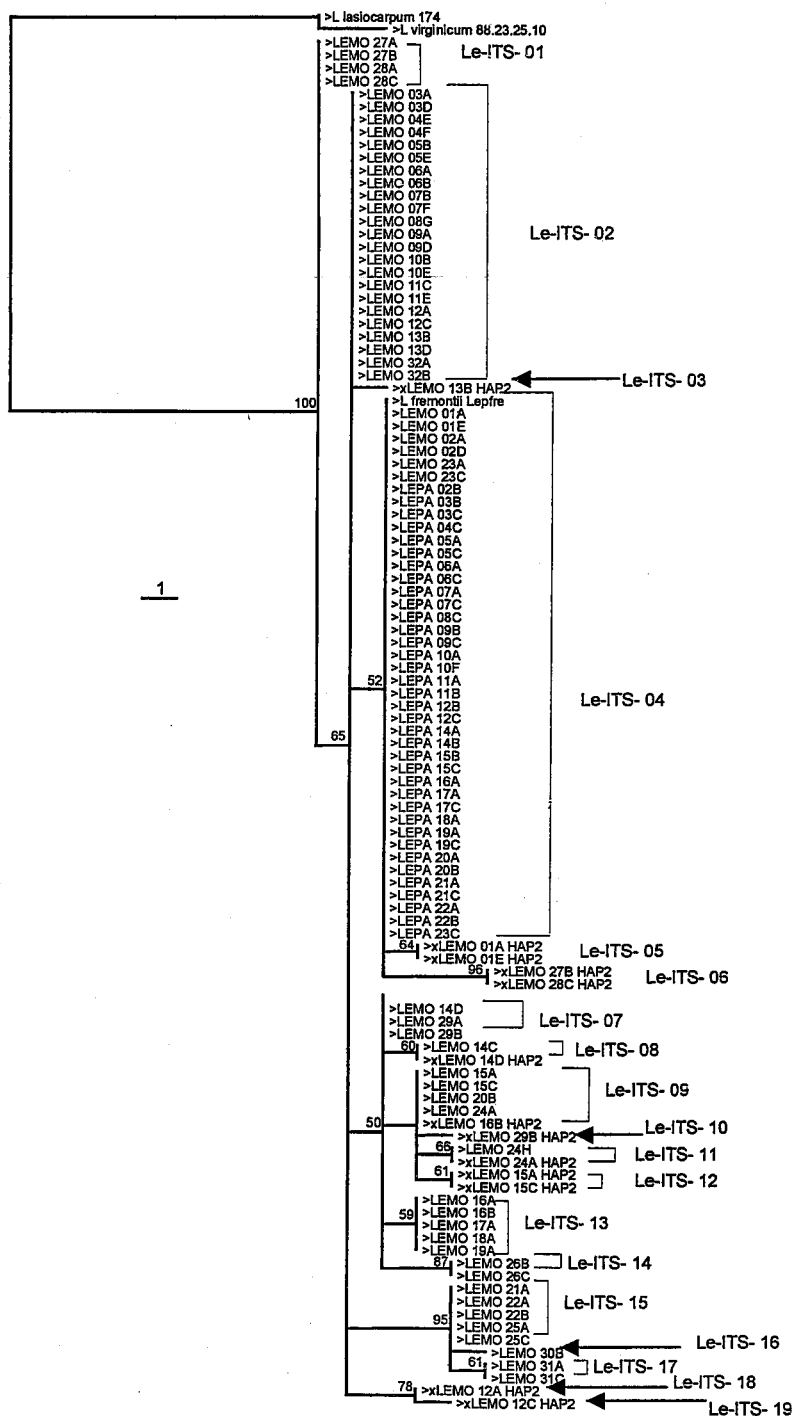


Figure 3-4 Phylogeny of *Lepidium montanum* (LEMO) and *L. papilliferum* (LEPA) inferred from nuclear ribosomal DNA internal transcribed sequence (ITS) haplotypes, obtained using a heuristic parsimony search, with bootstrap support values determined from 500 replicated samples. Shown is the single most parsimonious tree, with 49 steps (consistency index = 0.97, retention index = 0.99). Le-ITS codes refer to the 19 haplotypes which identical sequences condense.

Table 3-2 Frequency of ‘N’ numbers of individuals among *L. montanum* and *L. papilliferum* characterized by 19 ITS allelic haplotypes.

Haplotype:	<i>L. montanum</i>		<i>L. papilliferum</i>	
	N= (70)	Frequency	N= (36)	Frequency
Le-ITS_04	6	0.0857	36	1
Le-ITS_02	23	0.3286	0	0
Le-ITS_08	2	0.0286	0	0
Le-ITS_09	5	0.0714	0	0
Le-ITS_13	5	0.0714	0	0
Le-ITS_15	5	0.0714	0	0
Le-ITS_14	2	0.0286	0	0
Le-ITS_01	4	0.0571	0	0
Le-ITS_07	3	0.0429	0	0
Le-ITS_16	1	0.0143	0	0
Le-ITS_17	2	0.0286	0	0
Le-ITS_05	2	0.0286	0	0
Le-ITS_18	1	0.0143	0	0
Le-ITS_19	1	0.0143	0	0
Le-ITS_03	1	0.0143	0	0
Le-ITS_12	2	0.0286	0	0
Le-ITS_11	2	0.0286	0	0
Le-ITS_06	2	0.0286	0	0
Le-ITS_10	1	0.0143	0	0

the *L. montanum* and *L. papilliferum* taxa. Fifty-eight *Lepidium* accessions, including the 13 OTUs with mixed base calls, were simplified into 19 haplotypes in the ITS phylogeny (Figure 3-4). The frequency of individual samples characterized by each haplotype for either *L. montanum* or *L. papilliferum* is summarized in (Table 3-2). Haplotype ‘Le-ITS-04’ is comprised of all ITS *L. papilliferum* sequences (freq. = 1) as well as three *L. montanum* collection sites, namely ‘LEMO-01’, ‘LEMO-02’, and ‘LEMO-23’, and one Genbank *L. fremontii* accession. Haplotype ‘Le-ITS-05’, comprised of ‘LEMO-01A’ and ‘LEMO-01E’, differed from ‘Le-ITS-04’ by only one mixed-base site. The largest frequency (0.3286) of *L. montanum* collection sites are characterized by ‘Le-ITS-02’, with only one mutation difference from ‘Le-ITS-04’. Haplotypes ‘Le-ITS -01’, ‘-03’,

‘-05’, and ‘-07’, are separated from ‘Le-ITS-04’ by one to two mutation differences, where other haplotypes, ‘Le-ITS-08’ through ‘Le-ITS-19’, are separated from ‘Le-ITS-04’ by three to five character states.

Chloroplast DNA sequences- The UPGMA analysis of the chloroplast *trnL* intron and *trnL-F* intergenic spacer sequences also demonstrate relatively greater identity among *L. montanum*, *L. papilliferum*, and *L. fremontii* sequences, including those from GenBank (Mummenhoff et al., 2001) compared to other North American taxa (Figure 3-5) and all other *Lepidium* taxa. The *L. montanum* and *L. papilliferum* *trnL*-intron amplicons were 594 bp, including 40 bp of primer sequences. The overall alignment of *L. montanum*, *L. papilliferum*, and all other reference taxa, including 56 sequences available in GenBank (Mummenhoff et al., 2001), was 614 bp. Another 14 gap codes were included to account for indels. The *trnL* intron includes seven parsimony-uninformative and five parsimony-informative characters among the North American *L. montanum*, *L. fremontii*, and *L. papilliferum* taxa. Six of the latter parsimony-uninformative characters were unique to *L. fremontii*. Excluding *L. fremontii*, there were still five parsimony informative characters and one parsimony-uninformative character among the *L. montanum* and *L. papilliferum* taxa.

The LEMO and LEPA *trnL-F* spacer amplicons were variable in size, ranging from 515 to 598 bp, including 40 bp of primer sequence. The overall alignment of *L. montanum*, *L. papilliferum*, and all other reference taxa including 56 sequences available in GenBank (Mummenhoff et al., 2001), was 895 bp. Another 17 gap codes were included to account for relatively small simple indels. However, an internal 412-bp region of the alignment (positions 396-807) containing relatively large imperfect repeats

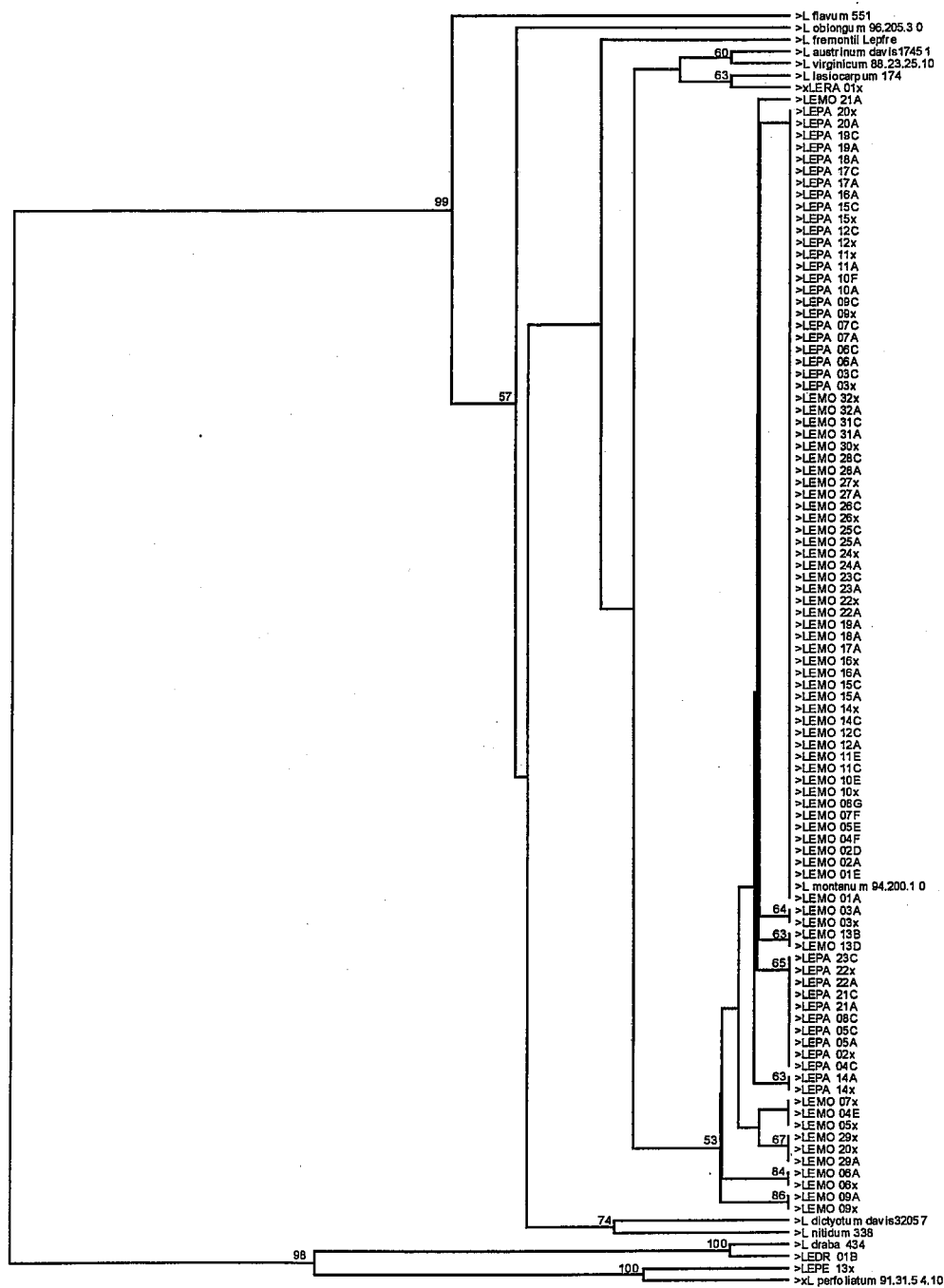


Figure 3-5 UPGMA distance analysis among *L. montanum* (LEMO), *L. papilliferum* (LEPA), and other North American *Lepidium* taxa based on the total number of differences (substitutions or indels) among the chloroplast *trnL* intron and *trnL-F* spacer DNA sequences, with bootstrap support values determined from 1000 sample reps.

was excluded from all analyses. The latter region accounts for much of the seemingly random size variation among our *L. montanum* and *L. papilliferum* samples, which we are currently assuming to be PCR artifacts. The *trnL-F* spacer region included two parsimony-uninformative and five parsimony-informative characters among the North American *L. montanum*, *L. fremontii*, and *L. papilliferum* taxa with no variable indel characters among these taxa. Both of the parsimony-uninformative characters were unique to *L. fremontii*, but the other five characters were parsimony-informative between *L. montanum* and *L. papilliferum*.

The combined *trnL* intron and *trnL-F* intergenic spacer had 28 parsimony-uninformative variable characters and 48 parsimony-informative variable characters among the North American taxa. Chloroplast DNA phylogenies consisted of 152 *Lepidium* OTUs. Figure 3-6 shows one of four equally parsimonious trees obtained using simple heuristic searches or a random sequence addition heuristic search with 100 replicates. The only differences among the four trees, relative to the one shown in Figure 3-5, involved 1) a collapse of the *L. oblongum* and *L. virginicum* clade into one trifurcated clade with *L. austrinum*, and 2) a synapse of the *L. fremontii* and *L. montanum* GenBank sequences (Mummenhoff et al., 2001) into one branch within the overall *L. montanum*, *L. papilliferum*, and *L. fremontii* clade. When *L. fremontii* was excluded, there was only one parsimony-informative and five parsimony-uninformative characters among the *L. montanum* and *L. papilliferum* sequences. A heuristic parsimony search found only one tree with six steps and no homoplasy among the *L. montanum* and *L. papilliferum* taxa, which is equivalent to the tree shown in Figure 3-4 minus other reference taxa and *L. fremontii*. There were no differences between most *L. papilliferum* and most

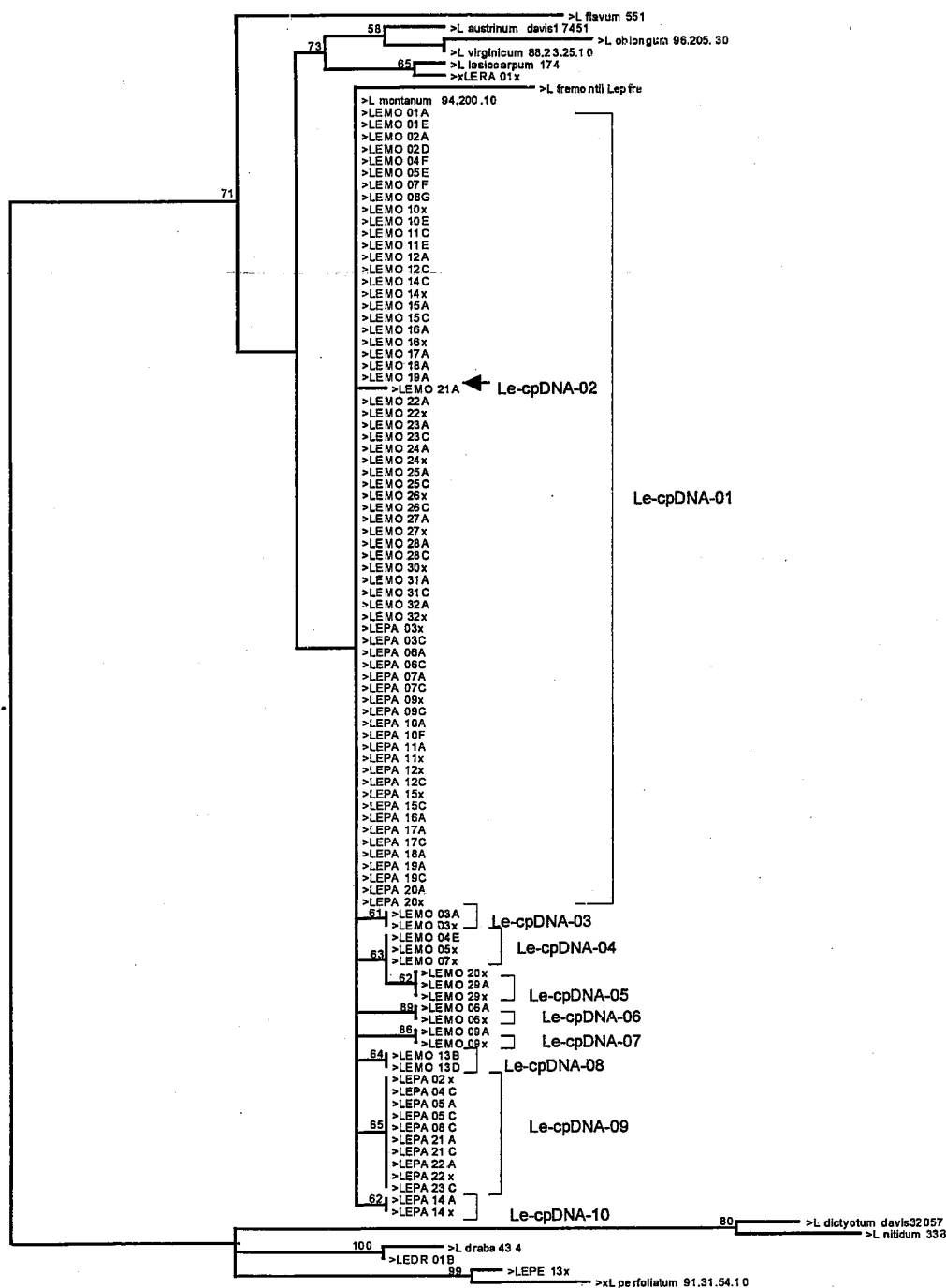


Figure 3-6 Phylogeny of *L. montanum* (LEMO), *L. papilliferum* (LEPA), and other North American *Lepidium* taxa inferred from the chloroplast *trnL* intron and *trnL-F* intergenic spacer DNA sequences, obtained using a heuristic parsimony search, with bootstrap support values determined from 500 replicated samples. Shown is one of four most parsimonious tree, with 86 steps (consistency index =0.86, retention index=0.85).

Table 3-3 Frequency of ‘N’ individuals among *L. montanum* and *L. papilliferum*, characterized by 10 chloroplast DNA allelic haplotypes.

Haplotype	<i>L. montanum</i>		<i>L. papilliferum</i>	
	N = (57)	Frequency	N= (36)	Frequency
cpDNA-Le-01	42	0.7368	24	0.6667
cpDNA-Le-03	2	0.0351	0	0
cpDNA-Le-04	3	0.0526	0	0
cpDNA-Le-06	2	0.0351	0	0
cpDNA-Le-07	2	0.0351	0	0
cpDNA-Le-08	2	0.0351	0	0
cpDNA-Le-02	1	0.0175	0	0
cpDNA-Le-05	3	0.0526	0	0
cpDNA-Le-09	0	0	10	0.2778
cpDNA-Le-10	0	0	2	0.0556

L. montanum sequences, including the *L. montanum* GenBank sequences (Mummenhoff et al., 2001). The frequency of individuals, either *L. montanum* or *L. papilliferum*, are characterized by ten haplotypes described in Table 3-3.

Lepidium papilliferum is characterized by three haplotypes, two of which ‘Le-cpDNA-09’ and ‘Le-cpDNA-10’ are loci unique to *L. papilliferum*. The two haplotypes separate from each other by two transition mutations and from the third *L. papilliferum* haplotype, ‘Le-cpDNA-01’, by one T-G transition mutation. ‘Le-cpDNA-01’ is shared between 13 *L. papilliferum* OTUs and 26 *L. montanum*. ‘Le-cpDNA-01’ characterizes the highest frequency (0.7368) of *L. montanum* and *L. papilliferum* (freq. =0.6667) collection sites. Of seven remaining *L. montanum* haplotypes, six separate from ‘Le-cpDNA-01’ by one transition mutation. ‘Le-cpDNA-05’ is separate by one C-G transversion in addition to a transition mutation, and ‘Le-cpDNA-04’ is separate by one C-G transversion only.

Divergence time estimates using calibrated molecular clock- Kimura’s 2-parameter average pairwise differences were calculated for the 19 ITS haplotypes and

10 cpDNA haplotypes derived from the heuristic parsimony analyses. K2P distance measures were analyzed in AMOVA to determine F-statistics (F_{st}) and the average pairwise differences within and between taxa. For ITS data, a significant difference ($F_{st} = 0.38107$) ($p = 0.00000$), with a corrected average pairwise difference of $(\text{PiXY} - (\text{PiX} + \text{PiY})/2) = 0.00128$, resulted between *L. montanum* and *L. papilliferum*, without consideration of varietal designations of *L. montanum*. For the cpDNA, a significant difference ($F_{st} = 0.11606$) ($p \leq 0.05$), with a corrected average pairwise difference of $(\text{PiXY} - (\text{PiX} + \text{PiY})/2) = 0.00008$, was reported between *L. montanum* and *L. papilliferum* alone, without consideration of varietal designations of *L. montanum*.

A divergence time of $2.5\text{-}5 \times 10^6$ (million) years ago (Mya) based on a minimum K2P sequence divergence of 1.8% (*trnT/L* spacer, *trnL* intron) and 4.4% (ITS), between *Rorippa* and *Cardamine* fossils (Brassicaceae), has been used to calibrate mutation rates between Australian and New Zealand *Lepidium* (Franzke et al., 1998; Mummenhoff et al., 2004). A 1% sequence divergence corresponds to 0.6-1.1 Mya for ITS and 1.3-2.8 Mya for the cpDNA. Sequence divergence between *L. montanum* and *L. papilliferum* was low, 0.124% in ITS and 0.008% in cpDNA. Using the same substitution rate calculated for *Rorippa* and *Cardamine*, we estimated the divergence between *L. montanum* and *L. papilliferum* to date to 136,000 to 74,000 ya for ITS and 22,400 to 10,400 ya for cpDNA. These time estimates correspond with paleoclimate records that show interglacial peaks of warmth (Berger, 1978; Watts, 1988) and pulses in speciation, approximately 120,000 ya and 10,000 ya. The Quaternary period, 1.8 million years ago to present, has been characterized by fluctuations in climate and environment corresponding to glacial-interglacial cycles. Scientists hypothesize that evolution among North American flora

during the past several million years has been markedly rapid, with pulses of macroevolution occurring every 100,000 years corresponding to glacial-interglacial climatic transitions (Stanley, 1979).

DISCUSSION

Comparisons among *Lepidium*, using the combined *trnL-trnL-trnF* spacer and the 18S-5.8S-26S nuclear ribosomal DNA internal transcribed spacer (ITS) regions, produced adequate polymorphic information to distinguish *L. montanum* and *L. papilliferum* from other North American *Lepidium*, including *L. fremontii*, *L. virginicum*, and *L. lasiocarpum*, among others in the phylogenetic analyses. Nuclear ITS sequences and chloroplast DNA sequence genotypes indicate that *L. papilliferum* is a geographically isolated form or extension of *L. montanum*. Haplotypes that characterize both *L. montanum* and *L. papilliferum* have the highest frequency among collection sites in both taxa in cpDNA and in *L. papilliferum* in the ITS sequences. Comparisons of several ITS haplotypes display more mutation differences within the *L. montanum* taxa than when compared with 'Le-ITS-04' that characterizes all *L. papilliferum*. The same observation is true for cpDNA *L. papilliferum* haplotypes 'Le-cpDNA-09' and 'Le-cpDNA-10', which are both differentiated from shared haplotype 'Le-cpDNA-01' by only one transition mutation, but separate from each other by two transition mutations.

'Identifying barcodes' clearly have the potential to distinguish *L. montanum* and *L. papilliferum* from other North American *Lepidium*. A preponderance of identical sequences in the cpDNA analysis suggests that molecular barcoding with the chosen PCR primers was not sufficient to distinguish the two taxa in the chloroplast region. The ITS

sequences on the other hand, produced one haplotype, 'Le-ITS-04', that was shared among all *L. papilliferum* and displayed potential to serve as an 'identifying barcode'. Three *L. montanum* accessions were also characterized by 'Le-ITS-04'. These *L. montanum* sites LEMO-01, LEMO-02, and LEMO-23, were either collected in relative proximity to the *L. papilliferum* collection sites, or possessed morphological characters similar to those that distinguish *L. papilliferum*. These findings, are in concurrence with results reported by Larson et al. (submitted), who performed amplified fragment length polymorphism analysis on the same data set, and showed consistent grouping between *L. montanum* accessions, LEMO-01, LEMO-02, and LEMO-23 and all *L. papilliferum* accessions. Identical sequences between *L. papilliferum* and *L. montanum*, may not necessarily point to a shortcoming of molecular 'barcoding' technique, but rather suggests that these two species are better categorized as the same species or that *L. papilliferum* should be considered a subgroup of the more widely distributed *L. montanum*. However, comparisons of average pairwise differences based on Kimura's 2-parameter test did reveal a significant difference between the two taxa. The sampling design may have created bias toward this statistic. The *L. papilliferum* Snake River Plain and Owyhee Plateau (Jarbidge) populations are two closely related and nearly panmictic populations.

The molecular clock estimates a recent divergence of between 136,000 and 74,000 ya (ITS) and 22,400 and 10,400 ya (cpDNA) between *L. montanum* and *L. papilliferum*. The Quaternary period, 1.8 million years ago to present, has been characterized by fluctuations in climate and environment corresponding to glacial-interglacial cycles. Scientists hypothesize that evolution among North American flora

during the past several million years has been markedly rapid, with pulses of macro-evolution occurring every 100,000 years, corresponding to glacial-interglacial climatic transitions (Stanley, 1979). More extensive geographical sampling of *L. montanum*, especially throughout the western range of distribution, and more intensive sampling of *L. montanum* in the Owyhee Plateau region will provide more insight into the evolution, natural history, and genetic relationships of *L. montanum* and *L. papilliferum*. Additional exploration of cpDNA barcodes with other candidate genes (Shaw et al., 2005) may also provide more insight into the relationship among *Lepidium* taxa.

As of January 2007, the proposal to list *L. papilliferum* as an endangered species was withdrawn (Fish and Wildlife FR Doc. 07–60, 2007) citing data that suggests the fluctuation in annual precipitation strongly correlates with changing abundance of the plant (Meyer and Allen, 2005; Palazzo et al., 2005; Menke and Kaye, 2006a). Other investigations showed no correlation between the abundance of *L. papilliferum* and total livestock penetration of the slickspot habitat (Menke and Kaye, 2006b), or the increased presence of weedy species and the abundance of *L. papilliferum*. Molecular data depicting a close genetic relationship between the isolated *L. papilliferum* and the more widespread *L. montanum* (Larson et al., submitted) was also cited as a factor in the decision to remove *L. papilliferum* from listing. Molecular barcoding techniques, incorporated with appropriate sampling could prove to be a practical means to determine relationships among other taxa of special concern when the Endangered Species Act merits an examination.

LITERATURE CITED

- ÁLVAREZ, I., AND J. F. WENDEL. 2003. Ribosomal ITS sequences and plant phylogenetic inference. *Molecular Phylogenetics and Evolution* 29: 417-434.
- BALDWIN, B. G., M. J. SANDERSON, J. M. PORTER, M. F. WOJCIECHOWSKI, C. S. CAMPBELL, AND M. J. DONOGHUE. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. *Annals of the Missouri Botanical Garden* 82: 247-277.
- BERGER, A. L. 1978. Long term variations of caloric insolation resulting from the earth's orbital elements. *Quaternary Research* 9: 139-167.
- BOWMAN, J. L., H. BRUGGEMANN, J-Y. LEE, AND K. MUMMENHOFF. 1999. Evolutionary changes in floral structure within *Lepidium* L. (Brassicaceae). *International Journal of Plant Science* 160: 917-929.
- DAVIS, R. J. 1952. in *Flora of Idaho* pp. 347. Brigham Young University Press, Provo, Utah, USA.
- DORN, R. D. 2001. *Vascular Plants of Wyoming*, 3rd ed. Mountain West Publishing, Cheyenne, Wyoming, USA.
- EXCOFFIER, L., P. E. SMOUSE, AND J. M. QUATTRO. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* 131: 479-491.
- FISHER, H., L. ESLICK, AND M. SEYFRIED. 1996. Edaphic factors that characterize the distribution of *Lepidium papilliferum* Technical Bulletin No. 96-6, Idaho Bureau of Land Management, Boise, Idaho. Pages 1-23.
- FRANZKE, A., K. POLLMANN, W. BLEEKER, R. KOHRT, AND H. HURKA. 1998. Molecular systematics of *Caradamine* and allied genera (Brassicaceae): ITS and non-coding chloroplast DNA. *Folia Geobotanica* 33: 225-240.
- HENDERSON, L. F. 1900. New plants from Idaho and from other localities of the Northwest. *Bulletin for the Torrey Botanical Club* 27: 342.
- HEWSON, H. J. 1981. The genus *Lepidium* L. (Brassicaceae) in Australia. *Brunonia* 4: 217-308.
- HITCHCOCK, C. L. AND A. CRONQUIST. 1973. *Flora of the Pacific Northwest*, pp. 170. University of Washington Press, Seattle, Washington, USA.

- HITCHCOCK, C. L., A. CRONQUIST, M. OWNBEY, AND J. W. THOMPSON. 1964. In *Vascular Plants of the Pacific Northwest*. pp 516. University of Washington Press, Seattle, USA and London, England.
- HOLMGREN, N. H., P. K. HOLMGREN, AND A. CRONQUIST. 2005. *Lepidium*. Pages 246-264. *Intermountain Flora, Vascular Plants of the Intermountain West, U.S.A.: Volume Two, Part B, Subclass Dilleniidae*. The New York Botanical Garden.
- KIMURA, M. 1981. Estimation of evolutionary distances between homologous nucleotide sequences. *Proceeding of the National Academy of Sciences of the United States of America*. 78: 454-458.
- KRESS, W. J., K. J. WURDACK, E. A. ZIMMER, L. A. WEIGHT, AND D. H. JANZEN. 2005. Use of DNA barcodes to identify flowering plants. *Proceedings of the National Academy of Science, USA* 102: 8369-8374.
- KUNTZE, C. E. O. 1891. *Revisio Generum Plantarum* 2: 937.
- LEE, J. -Y., K. MUMMENHOFF, AND J. L. BOWMAN. 2002. Allopoloidization and evolution of species with reduced floral structures in *Lepidium* L. (Brassicaceae). *Proceedings of the National Academy of Science, USA* 99: 16835-16840.
- MAI, D. H. 1995. *Tertiäre Vegetationsgeschichte Europas*. Fisher, Stuttgart, Germany.
- MENKE, C. A., AND T. N. KAYE. 2006a. *Lepidium papilliferum* (Slickspot peppergrass): Habitat Integrity Index Data Analysis (1998-2001). *Institute for Applied Ecology*.
- MENKE, C. A., AND T. N. KAYE. 2006b. *Lepidium papilliferum*, 1998-2004 Data Analysis Final Report. *Institute for Applied Ecology*.
- MEYER, S. E., AND P. S. ALLEN. 2005. *Lepidium papilliferum* Soil and seed bank characterization on the Orchard Training Area. U.S. Forest Service, Intermountain Research Station, Shrub Sciences Laboratory, Provo, Utah. Department of Plant and Animal Science, Brigham Young University, Provo, Utah.
- MUMMENHOFF, K., H. BRUGGEMANN, AND J. L. BOWMAN. 2001. Chloroplast DNA phylogeny and biogeography of *Lepidium* (Brassicaceae). *American Journal of Botany* 88: 2051-2063.
- MUMMENHOFF, K., E. KUHN, M. KOCH, AND K. ZUNK. 1995. Systematic implications of chloroplast DNA variation in *Lepidium* sections *Cardamon*, *Lepiocardamon* and *Lepia* (Brassicaceae). *Plant Systematics and Evolution* 196:75-88.
- MUMMENHOFF, K., P. LINDER, N. FRIESEN, J. L. BOWMAN, J.-Y. LEE, AND A. FRANZKE. 2004. Molecular evidence for biocontinentally hybridogenous genomic constitution in *Lepidium sensu stricto* (Brassicaceae) species from Australia and New Zealand. *American Journal of Botany* 91: 254-261.

- NELSON, A., AND J. F. MACBRIDE. 1913. Western Plant Studies. II. *Botanical Gazette* 56: 469.
- PALAZZO, A. J., LICHVAR, R. W., T. J. CARY, AND T. L. BASHORE. 2005. An analysis of the Seed Bank of *Lepidium papilliferum* (slickspot peppergrass). Unpublished report dated February 23, 2005 submitted to *Plant Ecology* in March 2005. 22 pp.
- ROLLINS, R. C. 1993. *Lepidium*. Pages 534-588. In: The Cruciferae of continental North America. Stanford: Stanford University Press.
- SHAW, J., E. B. LICKY, J. T. BECK, S. B. FARMER, W. LIU, J. MILLER, K.C. SIRIPUN, C. T. WINDER, E. E. SCHILLING, AND R. L. SMALL. 2005. The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *American Journal of Botany* 92:142-166.
- SOLTIS, P., D. SOLTIS, AND J. DOYLE. 1992. Molecular Systematics of Plants. Routledge, Chapman and Hall Inc. New York, New York, USA.
- STANFORD, A. M., R. HARDEN, AND C. R. PARKS. 2000. Phylogeny and biogeography of *Juglans* (Juglandaceae) based on *matK* and ITS sequence data. *American Journal of Botany* 87:872-882.
- STANLEY, S. M. 1979. Macroevolution. W. H. Freeman, San Francisco, California, USA.
- SWOFFORD, D.L. 2000. PAUP* Phylogenetic analysis using parsimony (*and other methods). Version 4.0b10. Sinaure Associates, Sunderland, Massachusetts, USA.
- TABERLET, P., L. GIELLY, G. PAUTOU, AND J. BOUVET. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* 17:1105-1109.
- THELLUNG, A. 1906. Die Gattung *Lepidium* (L.) R Br. Eine monographische Studie. Neue Denkschr Allg Schweiz Naturforsch Ges 41: 1-340.
- WATTS, W. A. 1988. In Vegetation History. pp. 155-192. B. Huntley and T. Webb III [eds.], Dordrecht, The Netherlands.
- WHITE, T. J., T. BRUNS, S. LEE, AND J. TAYLOR. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M.A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White. pp. 315-322. PCR protocols: a guide to methods and applications. Academic Press, San Diego, USA.

CHAPTER 4

CONCLUSION

The use of DNA barcodes as a standard technique for characterizing the biodiversity of the world's vascular plants is still in the exploratory stages. Several phylogenetic investigations have found that the *trnL-trnF*, the *trnH-psbA*, and other chloroplast regions provide varying levels of information among different taxonomic groups (Shaw et al., 2005; Kress et al., 2005). The most commonly sequenced locus among plants, the ITS region, has also displayed varying degrees of success in making species-level distinction among different taxa. The consensus among many authors (Comes and Abbott, 2001; Chase et al., 2005) is that a combination of nuclear and chloroplast region information is necessary to identify incidences of hybridization, introgression, and allopolyploidy in plants.

This thesis research attempted to delineate species-limits as well as the phylogenetic relationships among North American taxa of *Leymus* (Poaceae) and *Lepidium* (Brassicaceae) using the *trnL-trnF*, *trnH-psbA*, *trnK-rps16*, and the 18S-5.8S-26S nuclear ribosomal DNA internal transcribed spacer regions. Only two cpDNA loci were explored in *Leymus* (*trnH-psbA* and *trnK-rps16* spacers) and two cpDNA loci (*trnL* intron and the *trnL-trnF* spacer) were sequenced for *Lepidium* accessions. Both studies incorporated the 18S-5.8S-26S nuclear ribosomal DNA internal transcribed spacer regions. Species-specific 'barcoding' sequences were detected among three taxa in *Leymus*: *L. flavescens*, *L. innovatus*, and *L. mollis*. These three taxa also showed a high degree of within pairwise differences in ITS sequence data. Consistent with other plant

studies (Mason-Gamer et al., 1995; Sang et al., 1997; Comes and Abbott, 2001), a considerable number of polymorphisms were shared across species boundaries. Based on the loci explored in these two studies, the utility of the DNA barcodes as a means to identify an unknown plant specimen of *Leymus* or *Lepidium* to the species level is uncertain. Shared polymorphisms among species, especially among the two *Lepidium* taxa compared in cpDNA sequences, may question the legitimacy of their separate taxonomic designations. Although, one unique polymorphism shared across all *L. papilliferum* and three *L. montanum* with similar morphological traits, was observed in the ITS sequences. The preponderance of shared polymorphisms between species suggests hybridization, introgression, as well as several other confounding evolutionary events common to land plants, are likely to have occurred. Increased sampling with the use of other noncoding loci may reveal species-specific polymorphisms that would make possible the identification of an unknown specimen by means of DNA barcoding.

Phylogenies constructed by parsimony analyses reflect the low number of informative character differences between North American *Leymus*, as well as between North American *Lepidium*. The *Leymus* phylogenies are comprised of polytomies or branches separated by one or two mutational differences between North American taxa. One significant finding of the *Leymus* research was the distinct separation of North American and Eurasian *Leymus* in the cpDNA phylogeny. Eurasian *Leymus* also grouped closely with *Psathyrostachys juncea* the known diploid ancestor of *Leymus*. *Lepidium* topologies suggest *L. papilliferum* is very closely related to and may even be better classified as a subgroup of the widespread *L. montanum*. This is supported by the observation that the most frequently occurring haplotypes among OTUs characterized

both *L. montanum* and *L. papilliferum*.

Nucleotide sequence divergence among all *Leymus* taxa ranged from 0-1.9% in cpDNA, and 0-15.7% in ITS sequences. Sequence divergence between the two *Lepidium* taxa was 0.008% for cpDNA sequences and 0.124% for the ITS sequences. Molecular clock divergence estimates ranged from 650 000 ya to 2.3×10^6 mya (cpDNA) among North American and Eurasian *Leymus* species. Recent divergence estimates (133,000-74,000 ya (ITS), and 22,000-10,000 ya (cpDNA)) suggest a close relationship between North American *Lepidium* species.

The sampling design in both studies may have influenced divergence values within and among species. There were instances where *L. cinereus* had a higher total number of within-species differences than among other *Leymus*. The number of accessions per species varied widely in the *Leymus* study, from N=2 to N= 250, a discrepancy that may have impacted the average number of pairwise differences among *Leymus* taxa, the corresponding neighbor-joining trees (Figure 2-4 and 2-7, Chapter 2), as well as molecular divergence time estimates. The *Lepidium* study contained equal numbers of *L. montanum* and *L. papilliferum* accessions, yet a geographical bias among *L. papilliferum* accessions may have skewed the significant differences reported between them. Other closely related taxa contributed only one sequence or were totally excluded from the analyses. These taxa have similar habitat preferences and overlapping distribution with *L. montanum* and *L. papilliferum*. Their inclusion may have been useful to better establish the degree of relatedness among *L. montanum* and *L. papilliferum*.

Chloroplast and ITS sequencing techniques used in the two studies for this thesis research project have demonstrated some success in creating identifying barcodes to

determine a specimen to the species level. Inconsistencies between phylogenies developed from cpDNA and ITS sequences were observed in both taxa, suggesting the possibility of introgression and hybridization among other possible evolutionary events. Increased sampling with equitable representation among taxa, along with a larger number of cpDNA and nuclear regions tested, may improve the utility of DNA barcodes as a means to identify unknown specimens and construct accurate phylogenetic topologies.

LITERATURE CITED

- COMES, H. P., AND R. J. ABBOTT. 2001. Molecular phylogeography, reticulation, and lineage sorting in Mediterranean *Senecio* sect. *Senecio* (Astraceae). *Evolution* 55: 1943–1962.
- CHASE, M. W., N. SALAMIN, AND M. WILKINSON. 2005. Land plants and DNA barcodes: short-term and long-term goals. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 360: 1889–1895.
- KRESS J. W., K. J. WURDACK, E. A. ZIMMER, L. A. WEIGT, AND D. H. JANZEN. 2005. Use of DNA barcodes to identify flowering plants. *Proceeding of the National Academy of Sciences of the United States of America* 102: 8369–8374.
- MASON-GAMER, R. J., K. E. HOLSINGER, AND R. K. JANSEN. 1995. Chloroplast DNA haplotype variation within and among populations of *Coreopsis grandiflora* (Asteraceae). *Molecular Biology Evolution*. 12: 371–381.
- SANG, T., D. J. CRAWFORD, AND T. F. STUESSY. 1997. Chloroplast DNA phylogeny, reticulate evolution, biogeography of *Paeonia* (Paeoniaceae). *American Journal of Botany* 84: 1120–1136.
- SHAW J., E. B. LICKY, J. T. BECK, S. B. FARMER, W. S. LIU, J. MILLER, K. C. SIRIPUN, C. T. WINDER, E. E. SCHILLING, AND R. L. SMALL. 2005. The tortoise and the hare II: Relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *American Journal of Botany* 92: 142–166.