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ORIGINAL ARTICLE

Genetic structure of Eurasian and North American *Leymus* (Triticeae) wildryes assessed by chloroplast DNA sequences and AFLP profiles

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Abstract *Leymus* is a genomically defined allopolyploid of genus Triticeae with two distinct subgenomes. Chloroplast DNA sequences of Eurasian and North American species are distinct and polyphyletic. However, phylogenies derived from chloroplast and nuclear DNA sequences are confounded by polyploidy and lack of polymorphism among many taxa. The AFLP technique can resolve phylogenetic relationships between closely related species, with a curvilinear relationship expected between the proportion of shared bands and nucleotide substitution rate (*D*), up to about 0.100 *D*. The objective of this study was to compare *D* and phylogenetic relationships among 16 *Leymus* taxa, based on chloroplast DNA sequences and multilocus AFLP genotypes. Estimates of chloroplast *D* between taxa were 0.002 and 0.013 within and among continental regions, respectively. Estimates of AFLP *D* between taxa were 0.076 and 0.093 compared within and between continental regions, respectively, versus 0.024 within taxa. Bayesian and neighbor-joining cluster analyses effectively separated all AFLP genotypes by species, but showed that North American *L. ambiguus* is a hybrid species with nearly equal contributions from sympatric *L. cinereus* and *L. salinus* taxa. Two hierarchical AFLP clades, containing six North American taxa and four Eurasian taxa, had more than 98% bootstrap confidence with 0.071 and 0.055 *D* among taxa. Three other Eurasian taxa clustered with 79% and 89% confidence, with up to 0.79 *D* between taxa. These estimates provide benchmarks for phylogenetic comparisons of AFLP profiles, but three taxa could not be reliably grouped, which may reflect concurrent radiation of multiple lineages or lack of homologous AFLP characters caused by a high *D*.

Keywords Triticeae · Chloroplast · AFLP · *Leymus* · Nucleotide sequence divergence · Hybrid species

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Introduction

The genus *Leymus* encompasses about 30 perennial grass species from North America, South America, Europe, and Asia. *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 a segregate group of the tribe Triticeae, once assigned to *Elymus* (Bentham 1881; Hitchcock 1951). However, the perennial Triticeae grasses have been organized and divided into genomically defined genera, which show homologous chromosome pairing in pollen mother cells of interspecific hybrids (Dewey 1984; Löve 1984). The genus *Leymus* was initially defined by allotetraploid ($2n = 4x = 28$) species that showed 14 bivalents in pair-wise hybrids or seven bivalents plus seven monovalents when hybridized either to diploid *Psathyrostachys* (**Ns** genome) and diploid *Thinopyrum* (**J** genome) species (Dewey 1970, 1972, 1984; Löve 1984). Moreover, octoploid ($2n = 8x = 56$) and dodecaploid ($2n = 12x = 84$) forms likely arose via hybridization or nonreduced gametes

of the allotetraploid form (Dewey 1970; Anamthawat-Jonsson and Bodvarsdottir 2001), but otherwise share two distinct subgenomes similar to other *Leymus* taxa.

The presence of the *Psathyrostachys* **Ns** genome in *Leymus* has been repeatedly substantiated (Zhang and Dvorak 1991; Wang et al. 1994; Wang and Jensen 1994; Hole and Jensen 1999; Anamthawat-Jonsson and Bodvarsdottir 2001; Bodvarsdottir and Anamthawat-Jonsson 2003; Wu et al. 2003). However, early cytogenetic experiments raised doubt on the putative genome relationship between *Leymus* and *Thinopyrum*, which led to the currently accepted **NsXm** subgenome designations where **Xm** is from an unknown diploid ancestor (Zhang and Dvorak 1991; Wang and Jensen 1994). Studies of *L. mollis* and *L. arenarius* (Bodvarsdottir and Anamthawat-Jonsson 2003; Anamthawat-Jonsson 2005), and several other taxa (Zhang and Dvorak 1991), suggest that *Leymus* is a segmental autopolyploid derived from two distinct *Psathyrostachys* species with slightly different subgenomes ($\mathbf{Ns_1}$ and $\mathbf{Ns_2}$). However, two distinct nuclear ribosomal internal transcribed spacer (ITS) sequences (Liu et al. 2008, Sha et al. 2008) and two distinct sets of single-copy nuclear gene sequences (Fan et al. 2009; Wu et al. 2003; Larson et al. 2009) have been cloned and sequenced from the same *Leymus* wildrye plants including North American *L. condensatus*, *L. cinereus*, *L. triticoides*, and *L. innovatus* as well as Eurasian *L. chinensis*, *L. racemosus*, and *L. secalinus*. Sequencing of *Leymus* BAC clones containing homoeologous gene sequences showed that genomic DNA surrounding the conserved gene sequences is very different (Larson et al. 2009). Genetic mapping experiments demonstrated that genome-specific **Ns** and **Xm** markers show disomic inheritance (Wu et al. 2003; Larson et al. 2009) and are syntenous on **Ns** and **Xm** linkage groups (Wu et al. 2003). However, only **Ns**-like sequences have been detected in some taxa including *L. mollis* and *L. arenarius* (Liu et al. 2008; Sha et al. 2008). Although these nuclear gene studies (Liu et al. 2008, Sha et al. 2008; Fan et al. 2009) did not detect two distinct sequences from any of the *Leymus* taxa tested, some sequences may be present but not detected using PCR amplification, cloning, and sequencing, which yields somewhat inconclusive or contradictory results. For example, only one **Ns**-like sequence was cloned from *L. akmolinensis* in one study (Liu et al. 2008) and only one **Xm**-like sequence was obtained from *L. akmolinensis* in another study (Sha et al. 2008). Nevertheless, two distinct sets of *Leymus* nuclear gene sequences are consistently polyphyletic with respect to other Triticeae genera, where one set of sequences (**Ns**) is similar to *Psathyrostachys* and the other set of sequences (**Xm**) is similar to other Triticeae genera including *Agropyron*, *Thinopyrum*, *Pseudoroegneria*, *Hordeum*, *Triticum*, and *Secale* (Wu et al. 2003; Liu et al. 2008; Sha et al. 2008; Fan et al. 2009). Moreover, Jones et al. (1999) and Redinbaugh et al. (2000) showed that the chloroplast DNA sequences of North American *Leymus cinereus* and *Leymus triticoides* are very distinct from *Psathyrostachys*, which is generally accepted as one of the diploid ancestors of the polyploid genus, and significantly more similar to other Triticeae in general including *Agropyron*, *Australopyrum*, *Elymus*, *Pascopyrum*, *Pseudoroegneria*, and *Hordeum*.

These findings provide the first clear evidence that the chloroplast genome of North American *Leymus* taxa does not originate from *Psathyrostachys*. More extensive surveys of *Leymus* chloroplast DNA showed that most Eurasian *Leymus* wildryes are grouped with the diploid *Psathyrostachys,* the **Ns** genome donor, whereas most North American *Leymus* are grouped together in a clade distinct from Eurasian *Leymus* and *Psathyrostachys* (Culumber 2007; Liu et al. 2008; Zhou et al. 2010). The chloroplast *trnL-F* sequences of the North American *Leymus* taxa examined by Liu et al. (2008) are genetically similar to other Triticeae genera including *Hordeum*, *Aegilops*, *Secale*, *Pseudoroegneria*, and *Thinopyrum*, which is also consistent with chloroplast *ndhF* sequences (Jones et al. 1999; Redinbaugh et al. 2000). These findings suggest that the chloroplast DNA of North American *Leymus* taxa comes from the **Xm** ancestor of genus *Leymus*, whereas Eurasian *Leymus* taxa contain the **Ns** chloroplast genome from *Psathyrostachys* (Culumber 2007; Liu et al. 2008; Zhou et al. 2010). Thus, phylogenetic analysis of *Leymus* chloroplast DNA (Jones et al. 1999; Redinbaugh et al. 2000; Culumber 2007; Liu et al. 2008; Zhou et al. 2010) and nuclear gene sequences (Liu et al. 2008, Sha et al. 2008; Fan et al. 2009) have provided important evidence of differentiation and possible origins of the **Ns** and **Xm** subgenomes of allopolyploid *Leymus*. However, chloroplast and nuclear-gene sequences lack the polymorphism needed to distinguish species and are confounded by difficulties associated with polyploidy. Yang et al. (2008) analyzed 19 *Leymus* taxa from Eurasia and North America using RAPD markers. However, the RAPD markers did not resolve putative genetic differences between North American and Eurasian taxa, and statistical significance of the genetic variability within and among these 19 taxa and seven hierarchical groups was not demonstrated.

The amplified fragment length polymorphism (AFLP) technique (Vos et al. 1995) is a multilocus DNA fingerprinting method widely used to discriminate genotypes within plant species and has also been used to resolve phylogenetic relationships among closely related plant taxa (Aggarwal et al. 1999; Jones et al. 2008; Koopman et al. 2008; Zuriaga et al. 2009) including comparisons of different species within the Triticeae genera *Elymus* (Larson et al. 2003) and *Hordeum* (Pleines and Blattner 2008).

However, homoplasy caused by nonhomologous bands that show similar electrophoretic mobility and multiple ways in which AFLP bands can be lost cause ambiguities in phylogenetic reconstruction, especially when the proportion of homologous bands is low. Computer simulations show that the ratio of homologous to nonhomologous shared AFLP bands with equal size rapidly decreases as nucleotide DNA divergence (*D*) increases between zero and 0.10 *D*, but phylogenies are largely incorrect at *D* values above 0.05 (García-Pereira et al. 2010). The relationship between the proportion of shared AFLP bands and *D* is weak between 0.10 and 0.40 *D*, with no homologous AFLP bands expected beyond 0.4 *D* (García-Pereira et al. 2010). However, the probabilities of having nonhomologous bands with similar electrophoretic mobility is a function of the number of bands amplified and the range of band separation (Innan et al. 1999). The simulations conducted by García-Pereira et al. (2010) were based on conditions with an average of 90–100 bands of length between 72 and 472 bp, but did not account for differences in electrophoretic mobility between nonhomologous bands of the same length. Innan et al. (1999) developed methods to estimate *D* based on the proportion of shared AFLP bands, similar to methods developed for RFLPs (Nei and Li 1979), corrected for the probability of nonhomologous shared bands based on the number of bands, range of band separation, and multiple ways in which bands can be lost. Theoretically, the proportion of shared AFLP or RFLP bands is expected to have a curvilinear relationship with *D* from zero up to about 0.10 *D* (Innan et al. 1999; Nei and Li 1979), which is consistent with rates of homoplasy shown by García-Pereira et al. (2010). However, the methods developed by Innan et al. (1999) are difficult to implement and have been used in relatively few plant studies.

Estimates of *D* based on AFLP variation within Triticeae grass species vary from 0.005 to 0.020 within 11 different *Elymus* species (Larson et al. 2003) and up to 0.039 within *Pseudoroegneria spicata* (Larson et al. 2000) as determined using methods developed by Innan et al. (1999). Estimates of *D* based on AFLP or RFLP variation within genomically defined Triticeae genera range from 0.063 to 0.090 among tetraploid *Triticum* species (Mori et al. 1997) and 0.012 to 0.039 among tetraploid *Elymus* taxa (Larson et al. 2003). The maximum genetic distances among 13 North American and 2 Asian diploid *Hordeum* species correspond to *D* values approximately in the range 0.06–0.07 based on the proportion of shared bands as reported by Pleines and Blattner (2008). Likewise, estimates of *D* between diploid North American *Pseudoroegneria spicata* and four diploid Asian *Pseudoroegneria* species range from 0.06 to 0.10 based on the proportion of shared AFLP bands as reported by Larson et al. (2004). Therefore, estimates of nucleotide substitution rates (*D*) in four genomically defined Triticeae genera are within the range of detectable AFLP homology based on the methods of Innan et al. (1999). Thus, we speculate that the AFLP technique, optimized by reducing the number of bands and maximizing band resolution, may provide useful phylogenetic signals within other genomically defined polyploid Triticeae genera, including *Leymus*, which contain taxa that readily hybridize and show bivalent chromosome pairing. The objectives of this study were to use high-resolution multilocus AFLP profiles and chloroplast DNA sequencing side by side to (1) test and compare nucleotide sequence divergence within and between North American and Eurasian *Leymus* taxa, and (2) determine phylogenetic relationships among *Leymus* taxa.

Materials and methods

Plant materials included 97 individual plants representing 41 accessions of 16 *Leymus* taxa from North America, Europe, and Asia (Table 1). Replicate DNA samples from six plants were also included for AFLP analysis. The replicated T-tester *L. triticoides* genotype is one of three plants representing the same *Ltri*_Oregon (Acc641) *L. triticoides* accession (Table 1), but the replicated T-tester genotype was specifically labeled because it has been used as a parent of full-sib genetic mapping families (Wu et al. 2003). Reference samples of four Triticeae genera (*Agropyron*, *Hordeum*, *Psathyrostachys*, and *Thinopyrum*), *Poa*, and *Dactylis* were also included for chloroplast DNA sequence analysis (Table 1). Fresh leaf tissue was obtained from either the common garden established in North Logan, UT, or from seedlings grown in single-plant containers in the USDA-ARS, Forage and Range Research Laboratory greenhouse at Utah State University, Logan, UT. All leaf tissue samples were desiccated in a freeze dryer prior to DNA extraction. DNA was obtained from tissue of one or four individual plants per accession (Table 1) using a DNAeasy plant DNA isolation kit (Qiagen, Valencia, CA). The relative DNA content of most accessions was determined using the Partec PA ploidy analyzer (Partec, Münster, Germany) using tetraploid ($2n = 4x = 28$) 'Trailhead' and octoploid ($2n = 8x = 56$) 'Magnar' *L. cinereus* cultivars as standards.

AFLPs were assayed as described by Vos et al. (1995) with described modifications. A total of 103 individual DNA samples (including replicates) were preamplified with two selective nucleotides. Selective amplification primers consisted of two EcoRI+3/MseI+3 primer combinations and five *EcoRI+4/MseI+4* combinations, which were chosen based on preliminary testing of many primers to obtain profiles that had relatively simple but informative profiles. The stepwise addition of two selective nucleotides

Table 1 Description of plant materials including 97 plants representing 41 accessions of 16 *Leymus* taxa and other reference materials

Taxon	Sample identifier	Number of plants	Chromosome number	Origin	Germplasm accession number	Chloroplast DNA haplotypes	*trn*H-*psb*A Genbank accession	*trn*K-*rps*16 Genbank accession
Eurasian *Leymus* taxa								
L. akmolinensis	*Lakm*_Germany	3	28	Germany	PI 531794	01	EF485571	EF486215
L. angustus	*Lang*_Xinjiang1	3	84	Xinjiang, China	PI 565022 (D-3694)	53	EF485578	EF486214
	*Lang*_Xinjiang2	2	84	Xinjiang, China	(KJ-150)	53	EF485579	–
	*Lang*_Mustang	3	84	Saskatchewan, Canada	PI 271893		–	–
L. arenarius	*Lare*_Lithuania	2 (+1 replicate)	56	Lithuania	PI 531800	55	EF485580	EF486195
	*Lare*_Norway	2	56	Norway	PI 531801	55	EF485581	–
L. chinensis	*Lchi*_IMongolia1	3	28	Inner Mongolia, China	PI 499514	07	EF485582	EF485904
	*Lchi*_IMongolia2	2	28	Inner Mongolia, China	PI 499515	38	EF485583	–
L. mollis	*Lmol*_KrayRussia	3	28	Primorye Kray, Russia	(D-4086)	59	EF485855	EF486211
L. multicaulis	*Lmul*_XinjiangU	2	28	Urumqi, Xinjiang, China	PI 499521 (D-2694)	54	EF485856	EF486001
	*Lmul*_Shoshone	4	28	USA (naturalized)	(cv. Shoshone)	51	EF485881	EF486203
	*Lmul*_XinjiangW	3	28	Wujiaqi, Xinjiang, China	(D-3773)	54	EF485857	EF486177
L. racemosus	*Lrac*_PI565037	3	–	Botanical Garden, Berlin	PI 565037 (DJ-3801)	60	EF485862	EF486212
	*Lrac*_Kazakhstan1	1 (+1 replicate)	28	Kazakhstan	(JA-125)	53	EF485877	EF485991
	*Lrac*_Kazakhstan2	1 (+1 replicate)	28	Kazakhstan	(JA-129)	52	EF485878	EF486213
	*Lrac*_Volga.1	3	(28)	Russia	PI108491 cv. Volga		–	–
	*Lrac*_Volga.2	2	(28)	Russia	(Volga)		–	–
	*Lrac*_Volga.3	2	28	USA (naturalized Volga)	PI 531812	50	EF485863	EF486200
L. ramosus	*Lram*_Kazakhstan	2	28	Kazakhstan	PI 440332 (D-1775)	52	EF485865	EF486209
	*Lram*_Xinjiang	3	28	Xinjiang, China	PI 499653	52	EF485866	EF486210
L. secalinus	*Lsec*_Qinghai1	3	28	Qinghai, China	(D-3448)	38	EF485876	EF486194
	*Lsec*_Qinghai2	3	28	Qinghai, China	PI 504465	38	EF485879	EF486197
North American *Leymus* taxa								
L. ambiguus	*Lamb*_Colorado1	2	28	Fremont Co., Colorado, USA	(KJ-68)	02	EF485572	EF486216
	*Lamb*_Colorado2	3	28	El Paso Co., Colorado, USA	PI 531795 (D-3330)	35	EF485573	EF486180
	*Lamb*_Colorado5	2	28	Gilpin Co., Colorado, USA	W6 10226 (KJ-59)	04	EF485575	EF486219
	*Lamb*_Colorado7	2	–	Larimer Co., Colorado, USA	(KJ-54)	05	EF485576	EF486223

Table 1 continued

Taxon	Sample identifier	Number of plants	Chromosome number	Origin	Germplasm accession number	Chloroplast DNA haplotypes	*trn*H-*psb*A Genbank accession	*trn*K-*rps*16 Genbank accession
L. cinereus	*Lcin*_Alberta	2	28	Alberta, Canada	(Acc636)	43	–	EF486183
	*Lcin*_BColumbia	2	56	British Columbia, Canada	PI 598961 (T-1071)	41	EF485588	EF486206
	*Lcin*_Oregon	2	56	Oregon, USA	PI 537351 (T-18)		–	EF486185
	*Lcin*_Trailhead	2	28	Montana, USA	PI 478831 cv. Trailhead	43	EF485883	EF485922
	*Lcin*_Utah	2	56	Utah, USA	(U66-02)	41	EF485787	EF486218
	*Lcin*_Magnar	2	56	Saskatchewan, Canada	PI 469229 cv. Magnar	42 and 56	EF485849	EF485912
L. condensatus	*Lcon*_California1	1 (+1 replicate)	28 (56)	Atkins, California	PI 531804	41	EF485843	EF485907
	*Lcon*_California2	2	–	California		41	–	–
L. flavescens	*Lfla*_Idaho	3	28	Idaho, USA	(KJ-39)	31	EF485845	EF485917
L. innovatus	*Linn*_Alberta	3	28	Alberta, Canada	PI 236820	32	EF485848	EF485970
L. salinus	*Lsal*_Utah	2	28	Utah, USA	–		–	–
	*Lsal*_Wyoming	4	56 (28)	Green River, Wyoming, USA	PI 531815	35	EF485874	EF486188
L. triticoides	*Ltri*_Rio	2	(28)	Rio, California, USA	PI 490360 cv. Rio	46	EF485867	EF486182
	*Ltri*_Oregon	2	28	Jamieson, Oregon, USA	(Acc641)	41[a]	EF485861	EF485959
	T-tester	1 (+1 replicate)	(28)	Jamieson, Oregon, USA	(Acc641 T-tester)	41[a]	EF485860	EF485958
	*Ltri*_Nevada	1 (+1 replicate)	(28)	Humboldt Co. Nevada, USA	(Acc640)	41[a]	EF485887	EF486121
Agropyron								
A. cristatum	Kirk	1	(28)	Finland and other (Europe)	PI 536010 cv. Kirk		HQ286340	HQ286355
A. cristatum	Roadcrest	1	(28)	Turkey	PI 606546 cv. Roadcrest		HQ286348	HQ286363
A. cristatum ×	Hycrest	1	(28)	*A. cristatum* × *A. desertorum*	PI 549119 cv. Hycrest		HQ286339	HQ286354
A. derertorum	Nordan			USA (naturalized)	PI 469225 cv. Nordan		HQ286341	HQ286356
Hordeum								
H. vulgare	Steptoe	1	(14)		CIho 15229 cv. Steptoe		HQ286344	HQ286359
Psaythrostachys								
P. fragilis	PI343192	1	(14)	Iran	PI 343192		HQ286343	HQ286358
P. fragilis	PI401392	1	(14)	Iran	PI 401392		HQ286347	HQ286362
P. huashanica	PI 531823	1	(14)	Russia	PI 531823		HQ286342	HQ286357
P. juncea	Bozoisky	1	(14)	Kazakhstan	PI 440627 cv. Bozoisky		HQ286336	HQ286351
P. juncea	Mankota	1	(14)		PI 556988 cv. Mankota		HQ286337	HQ286352
P. juncea	PI 314521	1	(14)	Russia	PI 314521		HQ286346	HQ286361
P. lanuginosa	W6 26629	1	(14)	Kazakhstan	W6 26629		HQ286345	HQ286360
Thinopyrum								
T. elongatum	PI 531718	1	(14)	Tunisia	PI 531718		HQ286338	HQ286353

Table 1 continued

Taxon	Sample identifier	Number of plants	Chromosome number	Origin	Germplasm accession number	Chloroplast DNA haplotypes	*trn*H-*psb*A Genbank accession	*trn*K-*tps*16 Genbank accession
Poa								
P. pratensis	PI 317504	1		Afghanistan	PI 317504		HQ286349	HQ286364
Dactylis								
D. glomerata	PI 632497	1		Tunisia	PI 632497		HQ286350	HQ286365

Chromosome numbers inferred by ploidy analysis (or assumed from reported values). Germplasm accession numbers are from the USDA National Plant Germplasm System (or USDA Forage and Range Research Laboratory), with cultivar (cv.) names also provided.

[a] Treated as haplotype 44 by Culumber (2007), but difference from haplotype 41 was omitted from alignments used in this study.

during preamplification and one or two additional selective nucleotides for selective amplification ensured strict primer annealing with effective reductions in the number of amplified bands (Vos et al. 1995). The *EcoRI*-selective amplification primers included a fluorescent 6-FAM (6-carboxy fluorescein) label on 5′ nucleotides. Selective amplification products were combined with GS600 LIZ internal lane size standard and were fractionated using an ABI 3730 instrument with 50-cm capillaries and sized between 50 and 600 bp with Genescan software (Applied Biosystems, Foster City, CA). Although DNA molecules vary in length by increments of 1 bp, the relative mobility of bands is also affected by sequence composition. Thus, nonhomologous bands of the same length may not have the same relative mobility. Genescan trace files for each individual were visually analyzed for the presence or absence of DNA bands in bins that were at least 0.3 bp or more apart using Genographer software available free at http://hordeum.oscs.montana.edu/genographer/ or directly from the author, Tom Blake at blake@hordeum.oscs.montana.edu.

Genetic variability within and among taxa and geographic regions based on AFLP profiles of individual plants was quantified and tested using several techniques. Nei-Li genetic distances were calculated as $1-F$, where F is the proportion of shared bands between AFLP profiles of individual plants, as described by Nei and Li (1979). Nucleotide divergence (D) among taxa was estimated based on the average proportion of shared bands within and among taxa using a computer program, written in C, available at www.sendou.soken.ac.jp/esb/innan/InnanLab/Index_En.html (Innan et al. 1999). Bayesian clustering of individual plants without a priori assignment of individuals to hierarchical groups was used to determine genetic structure and test for possible admixture between taxa, which might otherwise have confounded phylogenetic analysis, using Structure v2.1 (Pritchard et al. 2000). Three analyses of each model with 100,000 iterations and 10,000 burn-in or 200,000 iterations and 20,000 burn-in with the dominant allele admixture model of Structure v2.2 (Pritchard et al. 2000; Falush et al. 2007). Different models of genotypic structure were compared using log probability, L(K), as described by Pritchard et al. (2000), and the change in log probability, ΔL(K), where K is the number of population groups (Evanno et al. 2005). Analyses were performed for all 16 *Leymus* taxa together, as well as separated into North American and Eurasian subgroups. Neighbor-joining cluster analyses of user-defined Nei-Li genetic distance and estimated nucleotide sequence divergence (D) matrices were performed using PAUP* (Swofford 2002). However, bootstrap support levels for groups in these trees were determined from 1,000 replicate searches using the restriction site distances (Nei and Li 1979) in

PAUP*, which is similar but not identical to the user-defined Nei-Li genetic distance or Innan's *D*. Analysis of molecular variance (AMOVA) was used to quantify and test genetic variability within and among taxa, continental provenances, and other empirically defined hierarchical groups based on estimates of *D*, number of DNA polymorphisms, and Nei-Li distances between AFLP profiles of individual plants using Arlequin (Excoffier et al. 1992).

The noncoding plastid chloroplast DNA regions were amplified by PCR using the *trnH-psbA* and *trnK-rps16* primers. Amplification of DNA sequences was performed in 25 µl volumes of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, and 0.1% Triton X-100, 2.5 mM $MgCl_2$, 2.0 mM DNTPs, 10 µM primers, 1 U *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 s), 53°C annealing (45 s), and 72°C extension (1 min 30 s) followed by 30 cycles of 94°C denaturing (30 s), 48°C annealing (45 s), and 72°C extension (1 min); 72°C extension (7 min). Quickstep 2 PCR and the ExcelaPure 96-well UF PCR purification kits (Edge BioSystems, Gaithersburg, MD) were used to purify PCR products prior to sequencing. Bidirectional sequencing reactions were performed using 0.25 µl of BigDye Terminator v3.1 cycle sequencing RR-100 reagent, 2 µl of BigDye Terminator v3.1 5× sequencing buffer, 1 µl of 2 µM primer, and 0.6 µl of purified PCR product in a 10 µl reaction volume as recommended by the manufacturer (Applied Biosystems, Foster City, CA), with the same primers used for PCR amplification. Products from the sequencing reactions were purified using a Performa DTR V3 96-well short plate kit (Edge BioSystems). The eluates were fractionated on an ABI3730 (Applied Biosystems) capillary electrophoresis instrument by the Utah State University Center for Integrated Biosystems. Complementary strands for each sample were aligned and manually inspected in SEQUENCHER 4.5 and 4.6 (Gene Codes Corporation, Ann Arbor, MI).

The chloroplast genome is inherited as a single unit without recombination. Thus sequences from the *trnH-psbA* and *trnK-rps16* amplicons were concatenated into a single consensus sequence for each sample (Soltis et al. 1996; McKenzie et al. 2006) and aligned using MEGA4 (Tamura et al. 2007). Simple alignment gaps were coded as indels, but some regions of poor alignment were excluded from the phylogenetic analysis as described in the Results. Phylogenetic analyses of the combined chloroplast *trnH-psbA* and *trnK-rps16* intergenic spacer haplotypes were performed using heuristic parsimony searches with simple sequence addition in PAUP*. Bootstrap support values for parsimony analyses were obtained from 1,000 heuristic searches with simple addition and a 50% consensus threshold. An AMOVA was also used to determine the percentage of chloroplast DNA variation within and between groups based on estimated nucleotide sequence divergence (*D*) using the K2P method (Kimura 1980).

Results

Comparisons of AFLP profiles within and between taxa

A total of 2,521 bins were scored over all seven AFLP primer combinations, with an average of 435 bins per AFLP primer combination. The smallest bin scored for each primer combination averaged 52 bp and the largest bin scored averaged 584 bp. Thus, scoring bins were an average of 1.22 bp apart. The average number of bands per plant was 231 over all seven primer combinations, with an average of 33 bands per plant per primer. The average smallest band was 65 bp and the average largest band was 539 bp when comparing each plant over all seven AFLP primers. Thus, there was an average of 14.4 bp between each band within any given profile (plant). However, in pair-wise comparisons of individual plants there was an average of 146 shared bands and 165 differences within taxa. Thus, there was an average of 44.4 bands spaced 10.7 bp apart in pair-wise comparisons of AFLP profiles within taxa. Similarly, there was an average of 57 shared bands and 348 differences in pair-wise comparisons of AFLP profiles among taxa. Thus, there was an average of 57.9 bands spaced 8.2 bp apart in pair-wise comparisons of AFLP profiles among taxa.

The total number of scored AFLP bands and the total number of monomorphic AFLP bands were compared within and among taxa relative to the number of plants and taxa sampled and the average number of bands per plant (Tables 2 and 3). The total number of bands scored within each species was positively associated with the number of plants sampled (Table 2). Octoploid ($2n = 8x = 56$) *L. arenarius* and dodecaploid ($2n = 12x = 84$) *L. angustus* displayed the largest number of bands per plant. Thus the number of bands detected in each taxa may also be affected by the number of chromosomes. The number of monomorphic bands was negatively associated with number of plants sampled for each species (Table 2). Thus, the percentage of monomorphic bands within taxa showed a strong negative association with the number of plants sampled (Table 2). Likewise, the number of bands conserved between taxa decreased, dramatically as the number of taxa being compared increased (Table 3). Thus, 32.9% of the 2,521 bands scored were present in only one taxon, 22.6% were present in two taxa, 44.5% were present in three or more taxa, but only 5% were present in nine or more taxa (Table 3). A total of 698 bands (27.7% of the total) were monomorphic in at least one taxon, but only

Table 2 Description of DNA profiles from 16 *Leymus* taxa using seven AFLP primer pairs

		Number of plants sampled	Number of bands scored	Number of monomorphic bands	Percent monomorphic bands	Average number of bands per plant	Average number of shared bands between plants	Average number of polymorphic bands between plants	Average Nei-Li[a] distance between plants	Average $D^b \times 100$ between plants
Averages within Eurasian taxa		6.1	467	97.6	23.9	233.2	151.9	162.6	0.350	2.27
01	*L. akmolinensis*	3	302	158	52.3	230.7	182.7	96.0	0.208	1.19
02	*L. angustus*	8	697	72	10.3	263.1	147.6	231.0	0.439	3.10
03	*L. arenarius*	4	425	165	38.8	267.2	203.2	128.0	0.238	1.42
04	*L. chinensis*	5	455	97	21.3	242.2	153.2	178.0	0.368	2.40
05	*L. mollis*	3	351	123	35.0	228.3	152.3	152.0	0.333	2.09
06	*L. multicaulis*	9	506	65	12.8	219.3	136.9	164.8	0.377	2.45
07	*L. racemosus*	12	609	51	8.4	216.6	130.6	172.0	0.397	2.62
08	*L. ramosus*	5	346	76	22.0	192.2	121.7	141.0	0.367	2.32
09	*L. secalinus*	6	515	71	13.8	239.3	138.9	200.8	0.421	2.88
Averages within North American taxa		6.0	511	81.6	21.1	228.5	138.6	167.6	0.388	2.64
10	*L. ambiguus*	9	761	47	6.2	252.7	126.6	252.1	0.499	3.74
11	*L. cinereus*	12	810	44	5.4	235.9	126.3	219.1	0.465	3.32
12	*L. condensatus*	3	354	110	31.1	224.8	153.5	142.5	0.317	2.00
13	*L. flavescens*	3	270	125	46.3	195.3	147.0	96.7	0.248	1.44
14	*L. innovatus*	3	341	117	34.3	222.0	147.3	149.3	0.339	2.14
15	*L. salinus*	6	556	66	11.9	248.7	135.6	226.1	0.455	3.24
16	*L. triticoides*	6	490	62	12.7	219.8	133.9	172.0	0.392	2.58
Averages within 16 taxa		6.1	487	90.6	22.7	231.1	146.1	164.6	0.366	2.43

[a] $(1 - F)$, where F is the proportion of shared bands (Nei and Li 1979)

[b] Average estimated number of nucleotide substitutions per site (D) based on proportion of shared AFLP bands, F (Innan et al. 1999)

Table 3 Frequency of AFLP bands conserved among *Leymus* taxa, classified as a function of the number of taxa compared

Number of taxa containing each band	All scored bands		Monomorphic bands		
	Number of bands	% of total bands	Number of bands	% of monomorphic bands	% of all (total 2,521) bands
1	830	32.9	460	65.9	18.2
2	570	22.6	104	14.9	4.1
3	402	15.9	47	6.7	1.9
4	230	9.1	22	3.2	0.9
5	141	5.6	16	2.3	0.6
6	108	4.3	11	1.6	0.4
7	53	2.1	5	0.7	0.2
8	59	2.3	6	0.9	0.2
9	39	1.5	8	1.1	0.3
10	15	0.6	2	0.3	0.1
11	11	0.4	3	0.4	0.1
12	19	0.8	2	0.3	0.1
13	11	0.4	3	0.4	0.1
14	7	0.3	3	0.4	0.1
15	11	0.4	3	0.4	0.1
16	15	0.6	3	0.4	0.1
Totals	2,521	100.0	698	100.0	27.7

238 (9.5% of the total) were monomorphic in two or more taxa (Table 3). A total of 1,691 bands were present in more than one taxa (Table 3) and 598 (35%) of these were monomorphic in at least one taxon. Most (86%) of the 698 monomorphic bands were present (if not monomorphic) in at least one other taxon. A total of 1,753 bands were scored among the nine Eurasian taxa, but only five bands were monomorphic among all nine taxa. A total of 1,906 bands were scored among the seven North American taxa, but only 11 bands were monomorphic among all seven taxa.

Although the total number bands and number of conserved bands among taxa was biased by sample size, chromosome number, and perhaps other factors, pair-wise comparisons of the number of conserved bands between taxa showed evidence of phylogenetic signal in pair-wise comparisons of taxa (Table 4). The average numbers of bands shared among taxa (and the percentage of bands shared between taxa) were 148.2 (18.7%) among pair-wise comparisons of Eurasian species, 170.1 (19.1%) among pair-wise comparisons of North American species, and 133.8 (15.6%) between North American and Eurasian species (Table 4). Similarly, the average numbers of monomorphic bands between taxa (and the percentage of shared bands that were monomorphic in both taxa) were 26.9 (19.8%) among pair-wise comparisons of Eurasian species, 26.4 (19.4%) among pair-wise comparisons of North American species, and 20.8 (17.8%) between North American and Eurasian species (Table 4). Overall, an average of 487 bands were scored within each species (Table 2), an average of 144.5 bands were shared between taxa (29.7% of the bands within species), and an average of 23.6 (16.3%) of the shared bands were monomorphic in both taxa (Table 2). Although pair-wise comparisons of the number and percentage of conserved bands (Table 4) provide useful descriptions and evidence of phylogenetic signal in the AFLP profiles, apparent by differences within and among geographic regions, these measures were affected by differences in sample size among taxa (Table 2).

Comparisons of the average number bands per plant and the average number of shared bands, the average number of polymorphic bands, the average Nei-Li distances, and the corresponding estimates of nucleotide divergence (D) based on pair-wise comparisons of AFLP profiles of individual plants (Tables 2 and 5) were independent of sample size within taxa. The average numbers of shared bands between AFLP profiles of individual plants was 146.1 within taxa (Table 2) compared to 63.3 among Eurasian taxa, 64.8 among North American taxa, and 57.0 between North American and Eurasian taxa (Table 5). The average number of polymorphic bands between individual plants was 164.6 within taxa (Table 2) compared to 339.9 among Eurasian taxa, 327.2 among North American taxa, and 360.4 between North American and Eurasian taxa (Table 5). The average Nei-Li genetic distances between AFLP profiles of individual plants was 0.366 within taxa (Table 2) compared to 0.730 among Eurasian taxa, 0.718 among North American taxa, and 0.781 between North

Table 4 Pair-wise comparisons of AFLP bands that were conserved among 16 *Leymus* taxa

		Eurasian taxa								North American taxa							
		01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16
01	*L. akmolinensis*	**302**	50	54	35	43	25	38	33	26	23	23	28	27	36	27	17
			25.2	*40.9*	*32.4*	*51.8*	*24.3*	*16.3*	*30.8*	*25.2*	*17.3*	*14.8*	*34.6*	*45.0*	*34.0*	*22.1*	*17.5*
02	*L. angustus*	198	**697**	41	28	27	21	37	31	15	21	24	25	21	26	24	18
		24.7		*16.8*	*16.1*	*19.3*	*11.0*	*8.8*	*19.0*	*7.5*	*8.2*	*8.1*	*18.4*	*18.9*	*15.5*	*11.4*	*11.5*
03	*L. arenarius*	132	244	**425**	30	57	24	46	29	21	25	30	31	33	37	31	23
		22.2	*27.8*		*22.6*	*40.4*	*19.4*	*14.0*	*23.8*	*16.5*	*15.4*	*14.5*	*32.0*	*45.8*	*29.1*	*22.6*	*20.5*
04	*L. chinensis*	108	174	133	**455**	30	15	21	25	22	20	20	22	22	29	24	17
		16.6	*17.8*	*17.8*		*26.5*	*11.3*	*8.4*	*25.3*	*14.2*	*10.4*	*8.2*	*20.4*	*28.6*	*25.4*	*14.0*	*14.4*
05	*L. mollis*	83	140	141	113	**351**	23	27	27	21	19	26	20	23	36	22	20
		14.6	*15.4*	*22.2*	*16.3*		*20.4*	*16.0*	*25.7*	*18.4*	*13.5*	*14.5*	*22.7*	*35.9*	*28.8*	*17.3*	*20.4*
06	*L. multicaulis*	103	191	124	133	113	**506**	21	17	12	16	21	16	17	21	17	14
		14.6	*18.9*	*15.4*	*16.1*	*15.2*		*9.7*	*14.3*	*7.8*	*8.9*	*9.2*	*15.1*	*20.2*	*18.3*	*11.0*	*10.2*
07	*L. racemosus*	166	297	236	178	131	175	**609**	24	14	16	23	19	22	24	17	17
		22.4	*29.5*	*29.7*	*20.2*	*15.9*	*18.7*		*12.6*	*5.7*	*5.9*	*6.8*	*10.4*	*20.7*	*13.2*	*7.3*	*8.5*
08	*L. ramosus*	107	163	122	99	105	119	151	**346**	17	19	20	20	19	24	21	16
		19.8	*18.5*	*18.8*	*14.1*	*17.7*	*16.2*	*18.9*		*13.1*	*12.7*	*11.4*	*22.7*	*30.6*	*22.9*	*17.2*	*15.0*
09	*L. secalinus*	103	201	127	155	114	153	174	130	**515**	16	15	14	18	18	17	10
		14.4	*19.9*	*15.6*	*19.0*	*15.2*	*17.6*	*18.4*	*17.8*		*8.6*	*6.6*	*13.5*	*24.0*	*16.8*	*10.8*	*8.5*
10	*L. ambiguus*	133	256	162	193	141	179	202	150	185	**761**	30	23	23	25	34	21
		14.3	*21.3*	*15.8*	*18.9*	*14.5*	*16.5*	*17.3*	*15.7*	*17.0*		*6.2*	*13.7*	*16.5*	*17.4*	*9.3*	*9.9*
11	*L. cinereus*	128	260	165	194	152	184	219	149	183	417	**810**	29	33	31	31	30
		13.0	*20.9*	*15.4*	*18.1*	*15.1*	*16.3*	*18.3*	*14.8*	*16.0*	*36.1*		*13.1*	*15.0*	*14.5*	*9.8*	*10.4*
12	*L. condensatus*	81	136	97	108	88	106	125	88	104	168	191	**354**	29	27	28	24
		14.1	*14.9*	*14.2*	*15.4*	*14.3*	*14.1*	*15.0*	*14.4*	*13.6*	*17.7*	*19.6*		*32.2*	*33.3*	*19.4*	*19.0*
13	*L. flavescens*	60	111	72	77	64	84	87	62	75	139	153	90	**270**	34	31	25
		11.7	*13.0*	*11.6*	*11.9*	*11.5*	*12.1*	*11.0*	*11.2*	*10.6*	*15.6*	*16.5*	*16.9*		*49.3*	*29.5*	*26.3*
14	*L. innovatus*	106	168	127	114	125	115	152	105	107	144	159	81	69	**341**	34	25
		19.7	*19.3*	*19.9*	*16.7*	*22.0*	*15.7*	*19.1*	*18.0*	*14.3*	*15.0*	*16.0*	*13.2*	*12.7*		*25.6*	*22.9*
15	*L. salinus*	122	210	137	172	127	154	178	122	157	364	265	144	105	133	**556**	25
		16.6	*20.1*	*16.2*	*20.5*	*16.3*	*17.0*	*18.1*	*15.6*	*17.2*	*38.2*	*24.1*	*18.8*	*14.6*	*17.4*		*14.1*
16	*L. triticoides*	97	156	112	118	98	137	130	107	118	212	231	126	95	109	177	**490**
		14.0	*15.1*	*13.9*	*14.3*	*13.2*	*15.9*	*13.5*	*14.7*	*13.3*	*20.4*	*21.6*	*17.5*	*14.3*	*15.1*	*20.4*	

Diagonal (bold): the total number of bands scored in each taxon. Below the diagonal: the number and percentage (italic) of scored bands present in both taxa. Above the diagonal: the number and percentage (italic) of shared bands that were monomorphic in both taxa

American and Eurasian taxa (Table 5). Finally, the average estimated nucleotide substitution rates (D) between AFLP profiles of individual plants were 0.024 within taxa (Table 2) compared to 0.078 among Eurasian taxa, 0.074 among North American taxa, and 0.093 between North American and Eurasian taxa (Table 5). Thus, the average nucleotide sequence divergence between continents, corrected for diversity within continents, is about 0.017 D based on the proportion of shared AFLP bands. AMOVA detected a highly significant variation ($P < 0.00001$) with approximately 9% of the variation apportioned between North American and Eurasian groups and 91% within continental regions (41% among taxa within continental regions and 50% within taxa) based on pair-wise comparisons of the average estimated number of nucleotide substitutions per site (D) between individual plants. Similar AMOVA results were obtained using the average number of polymorphisms and average Nei-Li distances among plants.

Comparisons of band homology and the estimated nucleotide substitution rates (D) between taxa provide evidence that AFLP profiles may be useful for empirically comparing the phylogenetics of *Leymus* taxa, for comparing Nei-Li distances and estimates of D among *Leymus*

Table 5 Pair-wise comparisons based on the average number of shared and polymorphic AFLP bands between individual plants of 16 *Leymus* taxa

		Eurasian taxa								North American taxa							
		01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16
01	*L. akmolinensis*	–	0.584	0.674	0.737	0.736	0.755	0.681	0.707	0.763	0.753	0.785	0.793	0.801	0.706	0.737	0.801
			4.83	*6.42*	*7.85*	*7.76*	*8.27*	*6.41*	*6.89*	*8.64*	*8.38*	*9.41*	*9.65*	*9.78*	*6.97*	*7.88*	*9.96*
02	*L. angustus*	102.6	–	0.619	0.72	0.75	0.742	0.599	0.704	0.762	0.765	0.775	0.783	0.809	0.708	0.759	0.798
		288.6		*5.46*	*7.50*	*8.33*	*8.04*	*5.03*	*6.94*	*8.78*	*8.96*	*9.23*	*9.47*	*10.39*	*7.14*	*8.74*	*10.08*
03	*L. arenarius*	81.5	101.4	–	0.752	0.655	0.773	0.602	0.725	0.783	0.76	0.776	0.791	0.799	0.716	0.752	0.801
		334.8	*327.5*		*8.49*	*6.03*	*9.08*	*5.09*	*7.47*	*9.60*	*8.81*	*9.30*	*7.34*	*9.96*	*7.26*	*8.52*	*10.25*
04	*L. chinensis*	62.1	70.9	63.2	–	0.743	0.787	0.77	0.771	0.737	0.759	0.776	0.789	0.812	0.75	0.742	0.806
		348.6	*363.6*	*383.0*		*8.01*	*9.46*	*8.81*	*8.70*	*7.89*	*8.63*	*9.14*	*9.58*	*10.37*	*8.19*	*8.08*	*10.28*
05	*L. mollis*	60.6	61.3	85.5	60.5	–	0.776	0.761	0.734	0.766	0.766	0.773	0.802	0.812	0.696	0.752	0.806
		337.9	*368.8*	*324.6*	*349.6*		*8.96*	*8.44*	*7.54*	*8.72*	*8.80*	*8.95*	*10.03*	*10.25*	*6.74*	*8.32*	*10.17*
06	*L. multicaulis*	55.1	62.3	55.2	49.3	50.1	–	0.786	0.77	0.778	0.788	0.793	0.817	0.8	0.759	0.782	0.795
		339.7	*357.8*	*376.1*	*363.0*	*347.4*		*9.24*	*8.55*	*9.10*	*9.57*	*9.65*	*10.66*	*9.65*	*8.35*	*9.30*	*9.63*
07	*L. racemosus*	71.4	96.3	96.7	52.7	53.3	46.5	–	0.73	0.79	0.804	0.798	0.815	0.806	0.737	0.797	0.825
		304.6	*287.1*	*290.5*	*353.4*	*338.4*	*342.9*		*7.39*	*9.54*	*10.24*	*9.84*	*10.54*	*9.89*	*7.70*	*9.89*	*11.02*
08	*L. ramosus*	61.9	67.4	63.3	49.8	56.0	47.4	55.2	–	0.764	0.774	0.773	0.795	0.824	0.745	0.779	0.803
		299.0	*320.5*	*332.8*	*334.8*	*308.5*	*316.7*	*298.4*		*8.46*	*8.87*	*8.74*	*9.47*	*10.52*	*7.81*	*9.02*	*9.76*
09	*L. secalinus*	55.7	59.9	55.2	63.4	54.6	51.1	47.9	51.0	–	0.784	0.804	0.816	0.821	0.78	0.782	0.821
		358.6	*382.6*	*396.2*	*354.8*	*358.4*	*356.5*	*360.1*	*329.5*		*9.54*	*10.29*	*10.79*	*10.78*	*9.19*	*9.44*	*11.02*
10	*L. ambiguus*	59.9	60.6	62.5	59.6	56.3	50.0	45.9	50.2	53.2	–	0.57	0.732	0.729	0.734	0.583	0.733
		363.6	*394.6*	*394.8*	*375.6*	*368.5*	*372.0*	*377.5*	*344.4*	*385.6*		*4.62*	*7.73*	*7.52*	*7.77*	*4.83*	*7.73*
11	*L. cinereus*	50.1	56.1	56.3	53.6	52.6	47.1	45.7	48.7	46.6	105.1	–	0.709	0.709	0.729	0.692	0.714
		366.4	*386.8*	*390.5*	*370.9*	*359.0*	*361.0*	*361.1*	*330.8*	*382.0*	*278.3*		*7.07*	*6.97*	*7.56*	*6.76*	*7.17*
12	*L. condensatus*	47.2	52.8	51.4	49.3	44.8	40.7	40.8	42.8	42.8	63.9	67.1	–	0.742	0.784	0.735	0.736
		361.1	*382.2*	*389.3*	*368.4*	*363.4*	*362.6*	*359.9*	*331.4*	*378.4*	*349.5*	*326.5*		*7.75*	*9.24*	*7.77*	*7.7*
13	*L. flavescens*	42.3	43.8	46.6	41.1	39.8	41.4	40.0	34.1	38.8	60.7	62.7	54.1	–	0.773	0.744	0.744
		341.3	*370.9*	*369.3*	*355.3*	*344.1*	*331.8*	*331.9*	*319.4*	*357.0*	*326.7*	*305.8*	*311.9*		*8.68*	*7.92*	*7.78*
14	*L. innovatus*	66.8	71.0	69.8	58.1	68.2	53.3	57.8	52.9	50.9	63.0	62.2	48.2	47.3	–	0.72	0.74
		319.1	*343.2*	*349.6*	*348.1*	*313.9*	*334.8*	*323.0*	*308.5*	*359.4*	*348.6*	*333.6*	*350.4*	*322.7*		*7.38*	*7.80*
15	*L. salinus*	62.9	61.8	64.0	63.4	59.2	51.1	47.3	48.7	53.1	105.3	74.5	62.8	56.9	65.9	–	0.727
		353.4	*388.3*	*387.9*	*364.1*	*358.7*	*365.7*	*370.8*	*343.5*	*381.7*	*290.7*	*335.5*	*347.8*	*330.1*	*338.9*		*7.56*
16	*L. triticoides*	44.8	48.7	48.5	44.9	43.5	45.1	38.2	40.7	41.0	63.2	65.2	58.7	53.0	57.3	63.9	–
		360.8	*385.5*	*390.1*	*372.2*	*361.0*	*348.9*	*360.0*	*330.6*	*377.0*	*346.1*	*325.2*	*327.1*	*309.0*	*327.1*	*340.6*	

Below the diagonal: average number of shared bands and the average total number of polymorphic bands (italic). Above the diagonal: average Nei-Li distance coefficients and average estimated *D* (italic)

[a] $(1 - F)$, where F is the proportion of shared bands (Nei and Li 1979)

[b] Average estimated number of nucleotide substitutions per site (D) based on proportion of shared AFLP bands, F (Innan et al.1999)

taxa and AFLP phylogenies described in other Triticeae genera (Larson et al. 2003; Pleines and Blattner 2008), and for investigating the theoretical relationships between the proportion of shared AFLP bands and D (Innan et al. 1999).

Cluster analyses of AFLP genotypes and genetic distances

The average model probability for the number of genotypic groups (K) increased gradually from $K = 1$ to $K = 12$ (Fig. 1), as determined by Bayesian cluster analysis of 97 plants (103 DNA samples) representing 16 *Leymus* taxa, but the model probabilities showed inconsistencies between different runs of this analysis when more complex models of population structure ($K \geq 8$) were evaluated. North American and Eurasian *Leymus* taxa effectively showed different ancestry in the simple two-group ($K = 2$) model test (Fig. 2a). However, several taxa including *L. innovatus*, *L. mollis*, and *L. chinensis* showed mixed ancestry coefficients in the $K = 2$ model. *Leymus*

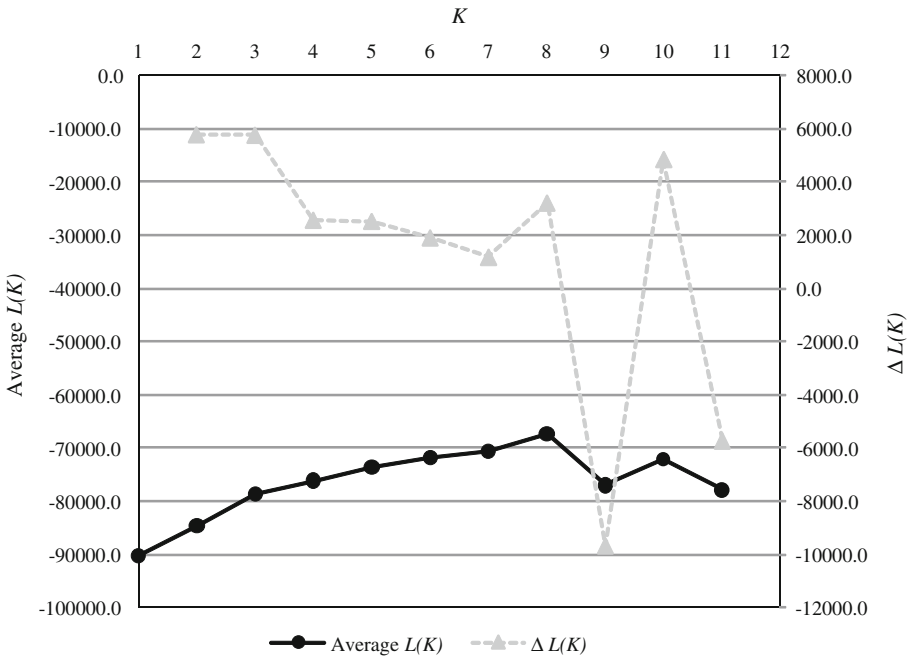

Fig. 1 Average model log probability, L(K), and change in model log probability, ΔL(K), for models with $K = 1$ to $K = 11$ groups (K being the number of groups tested) based on Bayesian cluster analysis of AFLP genotypes from 97 plants and six replicate DNA samples comprising 16 *Leymus* taxa

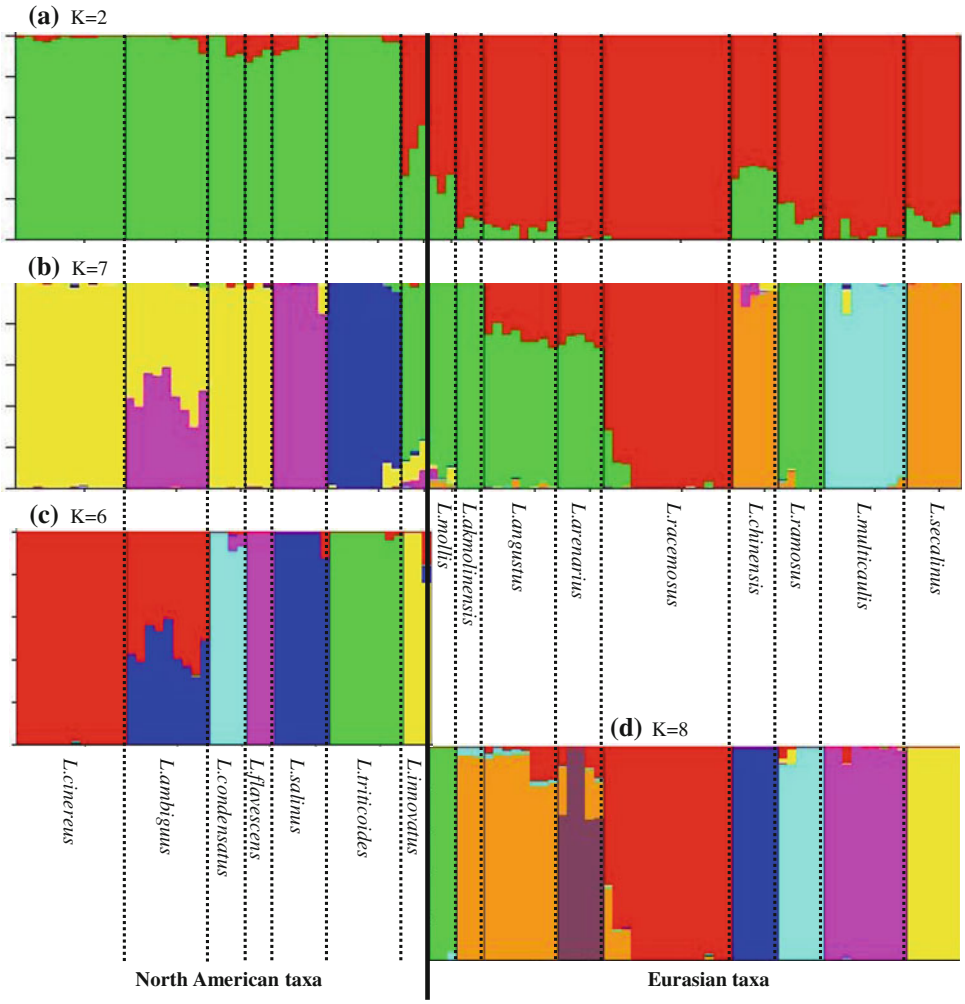

Fig. 2 Ancestry coefficients inferred from Bayesian model analysis of AFLP genotypes from 97 plants and six replicate DNA samples comprising 16 *Leymus* taxa: **a** two-group ($K = 2$) model test for all 16 taxa, **b** seven-group ($K = 7$) model test for all 16 taxa, **c** six-group ($K = 6$) model test for seven North American taxa, and **d** eight-group ($K = 8$) model test for nine Eurasian taxa

innovatus, in particular, had roughly equal North American and Eurasian ancestry coefficients. The Bayesian ancestry coefficient for the $K = 7$ model accounted for most of the structure when examining the entire germplasm collection from North America and Eurasia (Fig. 2b) with no apparent mixture of North American and Eurasian ancestry except for North American *L. innovatus,* which was similar to Eurasian *L. mollis* and *L. akmolinensis* (Fig. 2b). North American *L. innovatus* was similar to Eurasian *L. mollis*, *L. akmolinensis*, and *L. ramosus* (Fig. 2b). *Leymus multicaulis* displayed unique ancestry in models $K = 4$ through 7 with the exception of cultivar 'Shoshone', which showed slight admixture (12.1%) with North American cluster types (Fig. 2b).

Bayesian cluster analyses were performed separately for North American ($K = 2$ through 7) and Eurasian ($K = 2$ through 9) *Leymus* wildrye collections. Because the $K = 2$ Bayesian model test effectively separated the North American and Eurasian genotypes into different germplasm groups (Fig. 2), it seemed reasonable to simplify this test by performing separate analyses of these two major groups. The best models of genetic structure separated the seven North American taxa into six corresponding groups with an admixture of *L. cinereus* and *L. salinus* alleles in *L. ambiguus* (Fig. 2c). The hybrid origin of *L. ambiguus* was also evident in the $K = 7$ model for all 16 taxa (Fig. 2b, and other results not shown). The best models of genetic structure separated the nine Eurasian taxa into eight corresponding groups with *L. angustus* and *L. akmolinensis* in the same group (Fig. 2d). Moreover, some *L. arenarius* individuals showed unique and uniform ancestry, while others had as much as 30% mixed ancestry (Fig. 2d).

Neighbor-joining cluster analysis of AFLP genotypes representing 15 *Leymus* taxa clustered individual plants by locality (accession) and taxa with more than 90% bootstrap confidence, and detected two major hierarchical clades containing five North American taxa and four Eurasian taxa with more than 98% bootstrap confidence (Fig. 3). Nine plants representing the putative hybrid species *L. ambiguus* were excluded from this neighbor-joining analysis because it would have perturbed apparent relationships among *L. condensatus*, *L. flavescens* and *L. salinus*, and its parental ancestors, *L. cinereus* and *L. triticoides*, in this North American clade (Fig. 3). However, *L. ambiguus* did cluster with all five of these species in 100% of the bootstrap trees when all 97 plants (103 AFLP profiles), representing all 16 taxa, were analyzed (results not shown). The average estimated value of *D* among these six North American taxa (*L. ambiguus*, *L. cinereus*, *L. condensatus*, *L. flavescens*, *L. salinus*, and *L. triticoides*), which group together with 100% bootstrap confidence was 0.071. The average value of *D* among the four Eurasian taxa (*L. akmolinensis*, *L. angustus*, *L. arenarius*, and *L. racemosus*) that clustered together with 98% bootstrap confidence was 0.055. The latter Eurasian AFLP clade expanded to include a fifth Eurasian species, *L. ramosus*, with 79% bootstrap confidence with an average value of *D* of 0.072 compared with the other four taxa. Eurasian *L. chinensis* and *L. secalinus* clustered with 89% bootstrap confidence with an average value of *D* of 0.079 between these two taxa. A subclade of four North American taxa including *L. cinereus*, *L. condensatus*, *L. flavescens*, *L. salinus* and *L. triticoides* was also observed in 88% of the bootstrap trees (Fig. 3). Two collections of *L. racemosus* (JA-125 and JA-129, Table 1) were originally misidentified as *L. secalinus*, shown as *Lrac*_Kazakhstan1 and *Lrac*_Kazakhstan2 in Fig. 3.

Chloroplast DNA sequences

The *trnH-psbA* and *trnK-rps16* chloroplast DNA sequences collapsed into 22 *Leymus* haplotypes, including 17 haplotypes unique to only one *Leymus* taxon, four haplotypes (35, 38, 52, and 53) shared by two taxa, and one haplotype (41) shared by three taxa. Minor differences between haplotype 41 of *L. triticoides*, *L. cinereus*, and *L. condensatus* (Culumber 2007) were omitted from the sequence alignment used in this study. Thus, 28 unique species–haplotype combinations (taxonomic units) were analyzed (Fig. 4). Among the seven North American taxa, 11 chloroplast haplotypes were found, and 11 haplotypes were detected among the nine Eurasian taxa. With the exclusion of *L. mollis*, all haplotypes among the Eurasian taxa differed by only 1–3 bp mutations.

When combined, the *trn*H-*psb*A and *trn*K-*rps*16 chloroplast DNA amplicons had a total aligned sequence length of 1,339 bp and 20 gap codes among the 22 *Leymus* haplotypes and 15 reference sequences (Supplementary Data 1 and 2). Four regions of the sequence alignment (40–465, 686–705, 824–826, and 847–880) totaling 115 bp were eliminated because they contained complex indels and a palindromic rearrangement that complicated the sequence alignment (Supplementary Data 1). A total of 122 informative characters, including 68 parsimony-informative and 54 parsimony-uninformative, were analyzed among the 22 *Leymus* haplotypes and 15 reference sequences. The heuristic parsimony analysis resulted in 500 most parsimonious trees with 191 steps, a consistency index of 0.89, and a retention index of 0.93 (Fig. 4). The tree was rooted using non-Triticeae *Poa pratensis* and *Dactylis glomerata* sequences as outgroups (Fig. 4). The bootstrap support values shown above the branches were determined from 1,000 heuristic searches with simple sequence additions. The parsimony tree contained two main clades (Fig. 4). One clade, present in 80% of the bootstrap searches, included all North American *Leymus* taxa, one *L. mollis* sample from the Primorye Kray (Maritime Territory) of

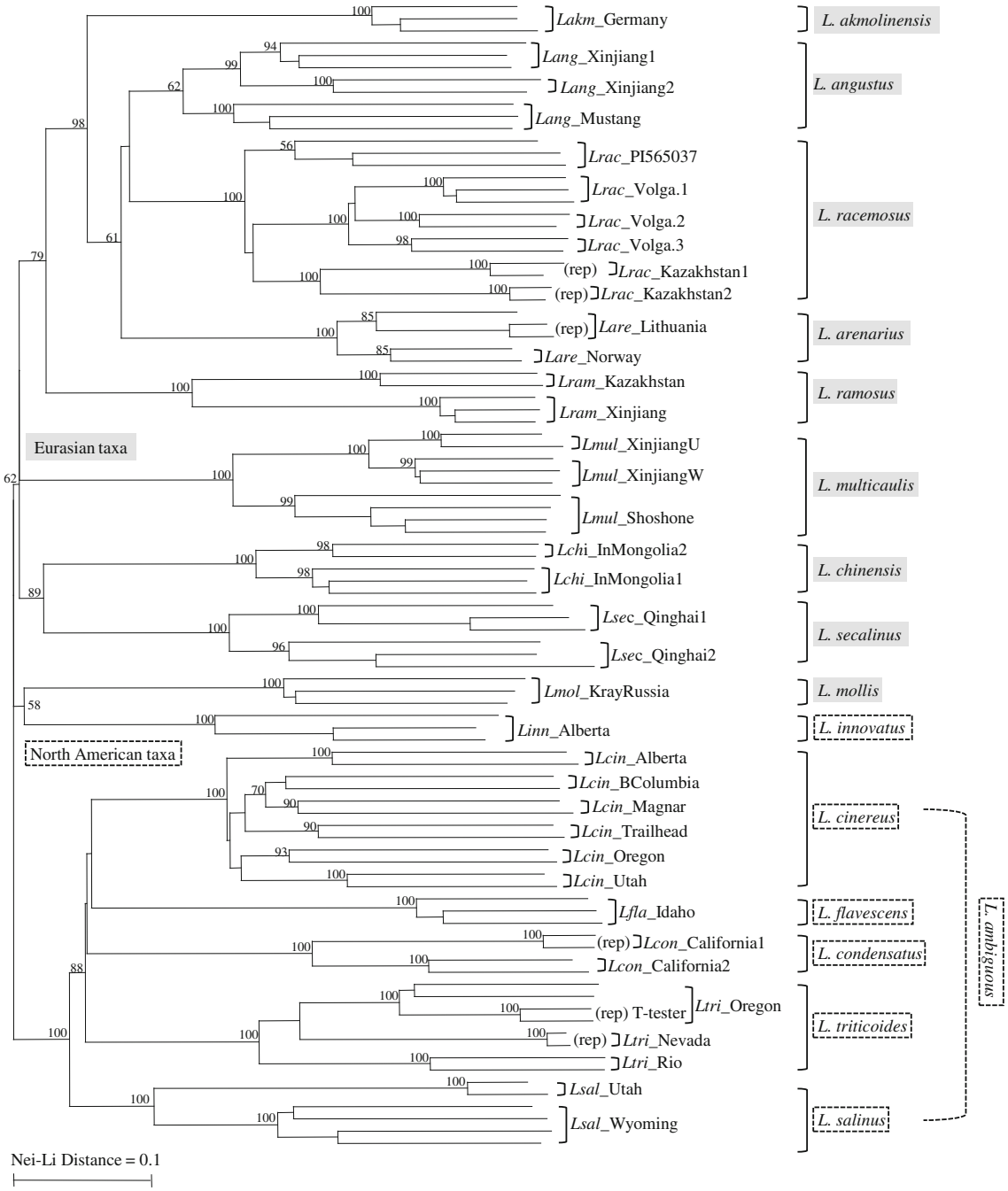

Fig. 3 Neighbor-joining cluster analysis based on pair-wise comparisons of Nei-Li distances between AFLP profiles of 88 individuals comprising 37 accessions of 15 *Leymus* taxa. Replicate DNA samples of six plants were also included for comparison. Nine plants representing *L. ambiguus*, a putative hybrid of *L. cinereus* and *L. salinus*, were excluded from this analysis. The frequency of each clade in the 50% majority rule consensus of 1,000 bootstrap resampling trees are given as percentages along the branches

Russia, near the Sea of Japan, in addition to all *Thinopyrum*, *Agropyron*, and *Hordeum* reference sequences. A second clade, found in 94% of the bootstrap searches, contained all remaining Eurasian samples (excluding the *L. mollis* sample from the Primorye Kray) and all of the *Psathyrostachys* reference sequences.

The average nucleotide substitution rates (D) within the North American and Eurasian chloroplast DNA clades were 0.003 and 0.001, respectively, whereas the average value of D between these clades was 0.013. Thus, the average nucleotide sequence divergence between continents, corrected for diversity within clades, is about 0.011.

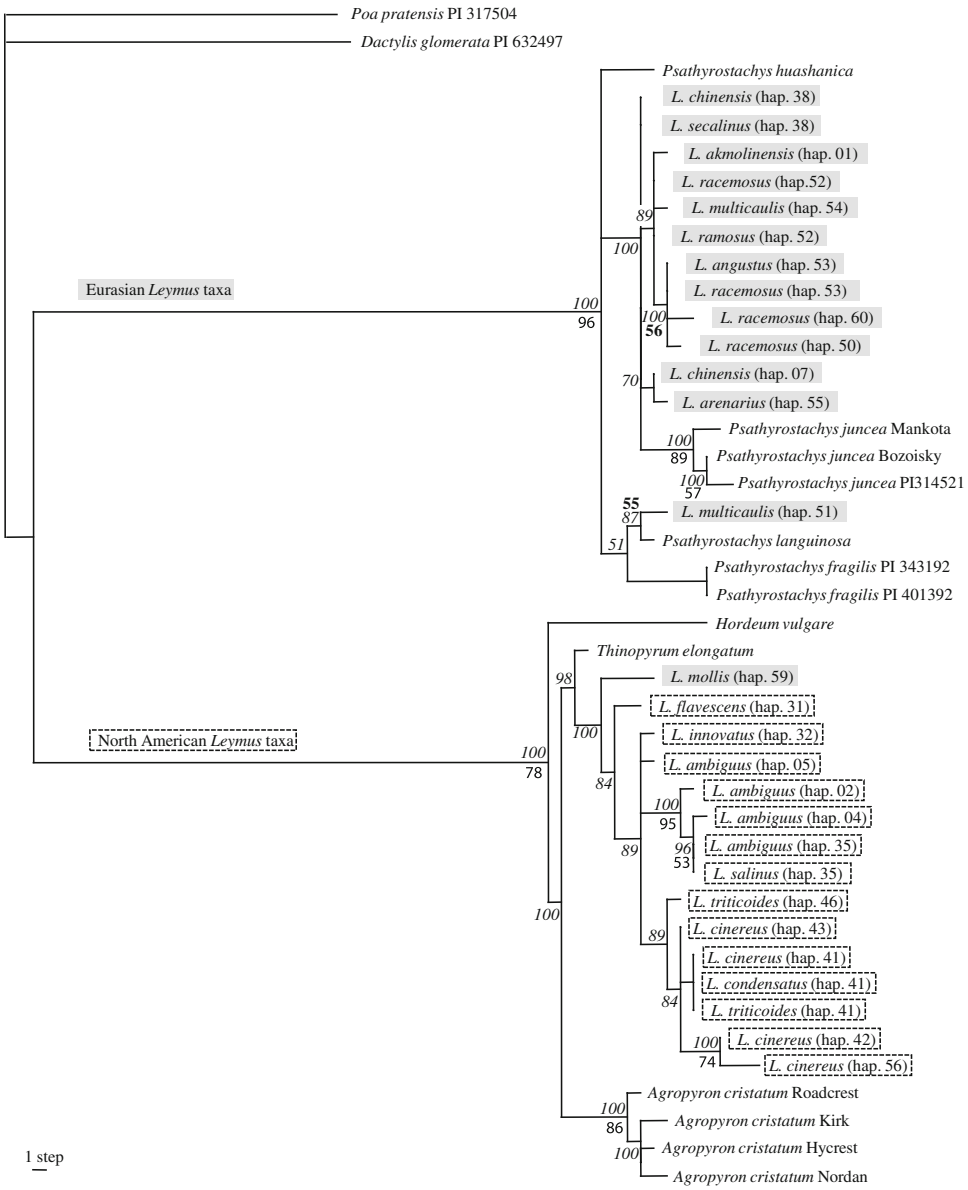

Fig. 4 One out of 500 equally parsimonious trees that is identical to the 50% majority rule consensus tree for 22 chloroplast haplotypes derived from sequences of the *trnH-psbA* and *trnK-rps16* spacers of 16 *Leymus* taxa (Table 1) with 68 parsimony informative and 54 parsimony uninformative characters. The 500 parsimony trees have a length of 191 steps with a consistency index of 0.89 and a retention index of 0.93. The frequency of each clade in the 50% majority rule consensus of 500 equally parsimonious trees (*numbers in italic*) and 1,000 bootstrap resampling trees (*numbers in bold*) are given as percentages along the taxa branches

The chloroplast DNA AMOVA showed that about 82% ($P < 0.001$) of the variation was apportioned between the Eurasian and North American clades of *Leymus*, 8% ($P < 0.001$) among taxa within clades, and 10% ($P < 0.001$) within taxa.

Discussion

The chloroplast DNA and AFLP markers displayed qualitatively similar but quantitatively different patterns of genetic variation. Both chloroplast and AFLP markers detected significant differences, by AMOVA, between North American and Eurasian taxa. However, about 82% of chloroplast DNA variation was apportioned between the Eurasian and North American chloroplast DNA clades, whereas approximately 91% of the AFLP variation was maintained within these geographic regions. Although rates of silent substitution in nuclear DNA genes is approximately double that of chloroplast DNA genes (Wolfe et al. 1987), cytoplasmic chloroplast DNA markers have a smaller effective population size and often detect greater differentiation between plant species, clades, and populations (Petit et al. 2005; Moyle 2006). Nevertheless, the estimated sequence divergence (*D*) between North American and Eurasian *Leymus* chloroplast DNA (0.011) was more than half of the corresponding value based on AFLP markers (0.017), when corrected for nucleotide diversity within these geographic regions. Moreover, the phylogeny of chloroplast *trn*H-*psb*A and *trn*K-*rps*16 DNA sequences used by Culumber (2007) and in this study demonstrated that North American and Eurasian *Leymus* are distinctly

different and polyphyletic relative to *Psathyrostachys* and other Triticeae genera, which is consistent with the findings of other studies based on the chloroplast *ndh*F locus (Jones et al. 1999; Redinbaugh et al. 2000), chloroplast *trn*L-*F* locus (Liu et al. 2008), and the chloroplast *rpo*A and *rbc*L loci (Zhou et al. 2010). The distinctiveness of North American and Eurasian *Leymus* chloroplast DNA was supported by bootstrap resampling (Fig. 4). Likewise, neighbor-joining cluster analysis of AFLP genotypes also detected two major hierarchical clades, which essentially distinguished six of the seven North American taxa from Eurasian taxa with 100% bootstrap confidence (Fig. 3). Two hierarchical AFLP clades containing six of the nine Eurasian taxa also had high bootstrap confidence (98% and 89%), and a seventh Eurasian species could also be clustered to a group of four other Eurasian taxa with 79% confidence. These Eurasian AFLP clades were also consistent with the chloroplast DNA phylogeny. For example, *L. chinensis* and *L. secalinus* grouped together in 89% of the AFLP dendrograms (Fig. 3) and shared an identical chloroplast haplotype that was different from other *Leymus* taxa (Fig. 4). The AFLP genotypes of two Eurasian taxa, *L. mollis* and *L. multicaulis*, and North American *L. innovatus* could not be reliably grouped, which may reflect concurrent radiation of multiple lineages (with short ancestral branches) and lack of homologous AFLP characters caused by high values of *D*. However, *L. multicaulis* displayed two divergent chloroplast DNA haplotypes and it was also unresolved from other taxa in the chloroplast DNA phylogeny (Fig. 4). Unresolved or poorly supported relationships were also observed within strongly supported clades of five North American taxa and four Eurasian taxa, which could also be the result of recent but concurrent radiation of multiple species with short ancestral branches. We speculate that there may have been rapid radiation of species following the formation and spread of allotetraploid *Leymus* throughout Eurasia and North America, which may be difficult to resolve into bifurcating branches. In any case, hierarchical AFLP clades were congruent with chloroplast DNA phylogenies, to the extent that these techniques detected phylogenetic relationships among these *Leymus* taxa.

The results of several studies (Culumber 2007; Liu et al. 2008; Zhou et al. 2010) including those reported here suggest that Eurasian and North American *Leymus* taxa, and the genus itself, originated independently by reciprocal hybridization of divergent **Ns** and **Xm** lineages. Although Eurasian and North American *Leymus* taxa contain similar **Ns** and **Xm** subgenomes, the **Ns** lineage was the female parent of the Eurasian *Leymus* taxa whereas the **Xm** lineage was the female parent of the North American *Leymus* taxa. Thus, patterns AFLP and chloroplast DNA variation, included in this study, are generally consistent with this hypothesis with several exceptions. The North American (**Xm**) chloroplast DNA genotype was found in *L. mollis* from the Russian Primorye Kray (Fig. 4) and from Alaska (Culumber 2007), whereas the Eurasian (**Ns**) genotype was also found in different *L. mollis* collections from Alaska (Liu et al. 2008) and China (Zhou et al. 2010). Thus, *L. mollis* carries divergent chloroplast DNA sequences, possibly representing the **Ns** and **Xm** ancestors, in Asia and North America. Moreover, the AFLP genotypes of circumpolar *L. mollis*, and boreal *L. innovatus*, also appear to be somewhat intermediate between North American and Eurasian AFLP clades (Figs. 2, 3). *Leymus innovatus* is very cold tolerant, colonizing grass adapted to high-latitude regions of North America, including the Aleutian Islands and other parts of the Bering region where *L. mollis* also occurs. Another exception to the genetic distinctiveness of North American and Eurasian *Leymus* wildryes involves the chloroplast DNA of *L. cinereus*. The chloroplast DNA sequences of North American basin wildrye (*L. cinereus*) samples used by Jones et al. (1999), Redinbaugh et al. (2000) Liu et al. (2008), Culumber (2007), and in this study (Fig. 4) were similar or identical to other North American taxa, whereas the *L. cinereus* chloroplast sequence reported by Zhou et al. (2010) was distinct from other North American taxa and more similar to *Psathyrostachys* and the Eurasian *Leymus* taxa. This exception is difficult to explain because Zhou et al. (2010) used the same *L. cinereus* cultivar 'Magnar' as that sequenced by Culumber (2007) and as used in this study (Table 1). However, Zhou et al. (2010) sequenced only one *L. cinereus* specimen whereas Culumber (2007) sequenced hundreds of *L. cinereus* accessions, including five reported here (Table 1, Fig. 4), all of which belong to the North American chloroplast DNA clade.

Bayesian analysis of AFLP genotypes revealed that *L. ambiguus* is a hybrid species, with roughly equal contributions of *L. cinereus* and *L. salinus* germplasm (Fig. 2). Similarities and differences between *L. ambiguus* and *L. salinus* have been recognized and examined (Atkins and Barkworth 1984). *Leymus ambiguus* populations are found primarily on the eastern slope of the Rocky Mountains from Montana south through Wyoming, Colorado and New Mexico (Atkins and Barkworth 1984). *Leymus salinus* occurs in eastern Utah, northern Arizona, southwestern Wyoming, and western Colorado. *Leymus cinereus* is widespread throughout high-elevation mountains, valleys, and basins of western North America. Thus, the distribution of *L. ambiguus* is within the extreme eastern range of *L. cinereus* and disjunctive (east) from the natural range of *L. salinus* (Atkins and Barkworth 1984), which suggests that this hybrid species has unique ecological adaptations that differentiate it from its putative ancestors. All four *L. ambiguus* accessions displayed different chloroplast

DNA haplotypes, three of which were identical or very similar to that of *L. salinus* (Fig. 4). Although these observations may suggest that *L. salinus* may have been the female ancestor of *L. ambiguus*, a similar pattern could have resulted from incomplete lineage sorting and persisting chloroplast haplotypes. More extensive sampling of all three taxa may reveal more complex patterns of genetic variation. In any case, it would not have been evident from the chloroplast DNA phylogeny (Fig. 4) that *L. salinus* is a hybrid species and it would have been difficult to detect and demonstrate this admixture using single-gene phylogenetic marker sequences such as the ribosomal ITS sequences. The AFLP technique provided important evidence for the evolution of a hybrid species, *L. ambiguus*, from *L. cinereus* and *L. salinus*.

In summary, the AFLP technique provided important new evidence of genetic differentiation and phylogenetic relationships among North American and Eurasian *Leymus* taxa, which were consistent with chloroplast DNA results, repeatable in bootstrap resampling, and more informative than chloroplast DNA at lower taxonomic levels. Moreover, comparisons of AFLP profiles between *Leymus* taxa have a direct theoretical relationship to nucleotide divergence (*D*) based on methods developed by Innan et al. (1999), which are comparable to other Triticeae genera. The average value of *D* in *Leymus* taxa, 0.024, was higher than in *Elymus* taxa, which is estimated to be between 0.005 and 0.020 (Larson et al. 2003), but less than in *Pseudoroegneria spicata*, which is estimated at 0.039 (Larson et al. 2000). The estimated average values of *D* among *Leymus* taxa were 0.076 and 0.093 within and between continental regions, respectively, with significant hierarchical clades detectable up to nearly 0.08 *D*. Thus, estimates of *D* among *Leymus* taxa were similar to corresponding estimates of 0.063–0.090 *D* based on RFLP among tetraploid *Triticum* species (Mori et al. 1997). Estimates of *D* among *Leymus* taxa were greater than the corresponding estimates of 0.012–0.039 *D* based on AFLP variation among North American and Eurasian *Elymus* taxa (Larson et al. 2003), and similar to estimates of 0.06–0.10 *D* based on AFLP variation between North American and Eurasian *Pseudoroegneria* taxa (Larson et al. 2004). The maximum Nei-Li distance of 0.66 observed among comparisons of New World and Asian *Hordeum* taxa (Pleines and Blattner 2008) corresponds to about 0.06–0.07 *D* based on the description of profiles reported in that study. The average Nei-Li genetic distances within and between Eurasian and North American taxa (about 0.72 and 0.78, respectively) were higher than those for *Hordeum*. Nevertheless, estimates of nucleotide substitution rates (*D*) in the genomically defined Triticeae genera, including *Leymus*, *Elymus*, *Hordeum*, *Pseudoroegneria*, and *Triticum* are within the range of detectable AFLP homology based on methods of Innan et al. (1999), especially if the number and resolution of the bands is optimized for these comparisons. Using methods developed by Innan et al. (1999) we found that the relationship between the proportion of shared AFLP bands and *D* deteriorated quickly above 0.1 *D* using relevant parameters from this study (results not shown). Although computer simulations have shown that phylogenetic trees based on the proportion of shared AFLP bands are largely inaccurate beyond 0.05 *D* (García-Pereira et al. 2010), the relationship between AFLP homology and *D* are dependent on the electrophoretic resolution and density of AFLP bands (Innan et al. 1999). The number and density of AFLP bands is a function of genome complexity, length of restriction enzyme recognition sequences, the number of selective nucleotides, and the overall electrophoresis separation. We used a two-step procedure with two selective nucleotides for AFLP preamplifications and another two arbitrary nucleotides during selective amplifications to reduce the genomic complexity of these *Leymus* taxa. We also used a high-resolution, capillary electrophoresis system, with a wide range (50–600 bp) of internal size standards that was capable of resolving homologous bands to a size difference of 1 bp and capable of detecting differences in relative mobility between nonhomologous bands that may have the same overall length (bp). Although homoplasy in AFLP datasets may cause incorrect tree topology when *D* is high and ancestral branches are short, false groups should not have significant bootstrap confidence if homoplasy is random. Methods described by Innan et al. (1999) provide a useful test of AFLP homology, which is partly conservative because it also assumes that nonhomologous AFLP amplicons with the same length would be scored the same.

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