Molecular variation in *Leymus* species and populations

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**Abstract**

Icelandic populations of European lymegrass [*Leymus arenarius* (L.) Hochst.] were examined using amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) of the major ribosomal genes (18S–5.8S–26S rDNA), in comparison with Alaskan populations of its closely related species *L. mollis* (Trin.) Pilger. The AFLP profiles emerged as two distinct entities, clearly separating the two species, and based on species-specific bands it was simple to distinguish these two morphologically similar species. The rDNA–RFLPs also differentiated the species. Within species, the Icelandic *L. arenarius* was more homogeneous than the Alaskan *L. mollis*, and its variation was dispersed over geographically different populations, suggesting a common gene pool. The variation among the Alaskan *L. mollis* was more extensive and its interrupted pattern may be the result of gene introgression at subspecies level. Within a 40-year-old population of *L. mollis* established in Iceland from Alaskan material, the molecular profiles separated old and new genotypes. Both AFLP and rDNA revealed the new genotypes to be extremely similar. This rapid change in allele frequency is thought to be the result of adaptation to a new environment.

**Keywords:** AFLP, *Leymus*, lymegrass, population analysis, rDNA, species differentiation

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**Introduction**

Two taxonomically related lymegrass species, North European *Leymus arenarius* (L.) Hochst. and North American/East Asian *L. mollis* (Trin.) Pilger, are among the most abundant perennial grasses of the Arctic and Arctic-temperate regions. The two lymegrass species are so similar in their morphology that they were once treated as subspecies of *Elymus arenarius* or *Leymus arenarius* (Bowden 1957; Hultén 1968; Hultén & Fries 1986). The only character that distinguishes them with certainty is chromosome number (*L. arenarius* is octoploid (2n = 8x = 56) and *L. mollis* tetraploid (2n = 4x = 28). However, the majority of lymegrass material can be identified on the basis of culm and spike characters (Sigurbjörnsson 1960; Barkworth & Atkins 1984). *L. arenarius* has glaucous culms and leaves, and the upper part of its culm is usually glabrous. In contrast, *L. mollis* foliage is generally green and the rachis is densely pubescent. The karyopsis size ranges of *L. arenarius* and *L. mollis* are nonoverlapping, the former being the larger (Anamthawat-Jónsson 1996).

Natural habitats of the two lymegrass species range from coastal to inland areas, including diverse soil types and climatic conditions. They are well adapted to cold climates and are often among the first species to colonize open areas. Due to extensive rhizomes, they have been widely used in Iceland and elsewhere for land reclamation purposes especially to stabilize erosion-prone soils (Klebesadel 1985; Greipsson & Davy 1994). Of the two species, *L. mollis* is generally the faster growing, at least in Icelandic conditions, and thus may be more suitable for land reclamation purposes (Helgadóttir 1993). Its soil-binding quality, together with its perennial habit, large seeds and tolerance to diverse environmental conditions, have also made lymegrass attractive as a potential crop for farming in marginal habitats (Anamthawat-Jónsson et al. 1994; Anamthawat-Jónsson 1996), or in a sustainable, multispecies, perennial system of future agriculture (Pimm 1997). In order to make full and effective use of the potential of lymegrass, it is important to assess the extent...
of genetic resources and the level of genetic variation that exist in these *Leymus* species.

Neither the genetics nor the molecular ecology of lymegrass has been much explored. A survey of leymin (seed storage protein) electromorphs of spatially separated *L. arenarius* populations from Southern Finland has demonstrated that the extent of genetic variation is greater between rather than within populations (Ahokas 1992). More generally, substantial variation for agronomic characters (seed yield, tillering, flowering time and lodging) has been noted among central Eurasian accessions of *L. racemosa* (Noel 1990). Current methods for assessing genetic diversity based on DNA polymorphism have yet to be applied to this species complex. In this study, we report the application of PCR-based fingerprinting and restriction fragment length polymorphism (RFLP) analysis to Icelandic populations of *Leymus* spp., both native (*L. arenarius*) and introduced (*L. mollis*), in comparison with Alaskan populations of *L. mollis*.

**Materials and methods**

**Plant materials**

The plant materials were collected as leaf tissue from 14 accessions of Icelandic *Leymus arenarius* and six accessions of Alaskan *L. mollis* (places of origin as shown in Table 1) in trial plots at Geitarsandi, Southern Iceland, established for 5 years. The plots consisted of rows of 20 seed-derived plants, spaced 1 m apart, with an inter-row distance of 1.5 m. The leaf samples were collected as pools of individuals, each sample representing a population/accession. To study variation within a natural population of *L. mollis* (IS1 at Múlakot, south Iceland), DNA was extracted from leaf samples collected from individual plants separated by 20 m, including eight plants from the population centre (G, old area) and eight from the periphery (N, new area). This population was established > 40 years ago with field-collected seeds from Alaska, and the population has expanded probably by rhizomatous tillering. The population is also sexually fertile and produces high seed yield. DNA extraction procedures were as described by Anamthawat-Jónsson & Heslop-Harrison (1993).

**Amplified fragment length polymorphisms (AFLP)**

Generation of AFLP fingerprints was achieved following Donini et al. (1997). Briefly, template DNA was double-digested with *Mse*I/*Sse*I, ligated to the appropriate adaptors and preamplified with the nonselective primers M00 and P00 (5'-GTAGACTGCGTACATGCAG-3' and 5'-GATGAGTCCTGAGTAA-3', respectively). Selective amplification was performed with a 32P-labelled P primer and an

<table>
<thead>
<tr>
<th><em>Leymus</em> species</th>
<th>Population</th>
<th>Origin</th>
<th>Location °N / °W (metres above sea level)</th>
</tr>
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<tbody>
<tr>
<td><em>L. arenarius</em></td>
<td>87.1</td>
<td>Iceland SW, Gardskagaviti</td>
<td>64.10/22.43 (&lt; 10)</td>
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<td><em>L. arenarius</em></td>
<td>87.3</td>
<td>Iceland SW, Sandvíkur</td>
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<td>Iceland NW, Hesteyri</td>
<td>66.22/21.50 (&lt; 10)</td>
</tr>
<tr>
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<td>Island SW, Seltjarnarnes</td>
<td>64.10/22.00 (&lt; 10)</td>
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<td>65.43/19.35 (1–100)</td>
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<td>63.50/17.30 (1–100)</td>
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<td><em>L. arenarius</em></td>
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<td>Iceland, Sandodd</td>
<td>65.35/24.10 (&lt; 10)</td>
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<td>Alaska, Kotzebue</td>
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<td>64.27/164.49 (&lt; 10)</td>
</tr>
<tr>
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<td>The same location as A061 A</td>
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<td>60.13/154.20 (100)</td>
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<td>IS-1 (G &amp; N)</td>
<td>Iceland S, Múlakot</td>
<td>63.48/20.05 (&gt; 100)</td>
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unlabelled M primer using combinations of P00-A, P00-C, P00-TC, P00-GAC, P00-GTG, P00-GTT with M00-A, M00-G, M00-T, M00-CG and M00-CTC. Labelled PCR products were separated on 6% denaturing polyacrylamide gels and the fingerprints were captured on a PhosphorImager.

Distinct and polymorphic AFLP bands from a total of 250 band positions across the 36 Leymus samples, generated by four primer sets, were recorded as present (1) or absent (0). Genetic distance analysis was carried out on these primary data using the program RAPDISTANCE (Armstrong et al. 1996), based on pairwise similarities calculated as follows (Dice 1945; Nei & Li 1979):

\[ 2n_{11}/[(2n_{11}) + n_{01} + n_{10}] \]

Where: \( n_{11} \) is the number of band positions at which sample x is 1 and y is 1, \( n_{01} \) is the number of band positions at which sample x is 0 and y is 1, and \( n_{10} \) is the number of band positions at which sample x is 1 and y is 0. The resulting similarity matrix was used for constructing a neighbour-joining tree.

**rDNA–RFLP**

The 9-kb fragment of ribosomal genes (18S–5.8S–26S rDNA) isolated from wheat (clone pTa71, Gerlach & Bedbrook 1979) was used as probe in Southern hybridization of BamHI-restricted DNA of the same lymegrass samples analysed by the AFLP, from both the Icelandic *L. arenarius* and the Alaskan *L. mollis*. The chemiluminescence Southern hybridization was performed as by Anamthawat-Jónsson & Heslop-Harrison (1993).

**Results**

The AFLP profile of the lymegrass samples consisted of 50–100 discrete bands, depending on the primer combination employed. The two species, *Leymus arenarius* and *L. mollis*, were readily distinguishable from one another, irrespective of the primer combination, because a number of bands was specific to only one of the two species (Fig. 1) and these appeared in the profile of all the samples of the respective species. The extent to which *L. arenarius* and *L. mollis* both vary and are conserved at the AFLP level allowed the species to be clearly differentiated by the pairwise distance analysis based on polymorphic bands (Fig. 2). The AFLP analysis placed one *L. arenarius* sample (accession 86.14) in the *L. mollis* group. From visual inspection of several AFLP gels, profiles of this DNA clearly included *L. mollis*-specific bands and lacked *L. arenarius*-specific bands. The original *L. arenarius* plants in the relevant plot were noted to have grown poorly in the 2 years prior to sampling, consistently across replicates (Helgadóttir 1993), and we conclude that the leaf sample taken for DNA analysis originated from an invading *L. mollis* plant from the neighbouring row (Alaskan accession A106, not included in the analysis).

The overall level of polymorphism within both species was low, which made AFLP useful for species identification as well as for detection of misclassification. In particular, the profiles of different accessions of the Icelandic *L. arenarius* were very similar (see, for example, Fig. 1). The distance analysis (Fig. 2) placed the *L. arenarius* accessions together (branch group 1) and without distinct association to geographical locations. For example, accessions from southwest Iceland including the Reykjanes Peninsula (87.1, 87.3, 87.4, 86.1, 86.5) were dispersed within the species group, whereas some of these southwest accessions seemed to be more related to the northernmost accessions (87.8, 86.9) than to other southwest accessions. Nevertheless, the analysis detected relatedness among accessions from the same region with similar climates, for example between northern accessions 86.3 and 86.9, and between eastern accessions 86.8 and 86.11. *L. arenarius* is the only native lymegrass species in Iceland, and as a whole its AFLP variation appeared to be continuous among the samples collected from all around the country.

The AFLP profiles among the accessions of *L. mollis* from Alaska were relatively more polymorphic than those of *L. arenarius* in Iceland, regardless of primer combinations used. The genetic distance analysis (Fig. 2) revealed the average branch length among the *L. mollis* accessions (branch group 3) to be twice as long as that among the *L. arenarius* entries (branch group 1). The means of relative phenetic distances were 0.144 (range 0.106–0.196) for *L. mollis* and 0.076 (range 0.03–0.138) for *L. arenarius* (actual data not shown in Fig. 2). This large variation did not seem to be associated with geographical distances or locations; thus two nearby populations from the Seward Peninsula (A061A and A061B) were more different from one another than they each were from population A204, which was obtained from a distant location in the Alaska Peninsula.

The AFLP method is clearly also robust for identification at the subpopulation level. The profiles of individual plant samples in the Icelandic population of Alaskan *L. mollis* (IS1: G and N locations) were unexpected in that they fell into two distinct groups corresponding exactly to their locations: the old G-group in the population centre and the new N-group from the population edges (Fig. 2, branch group 4). The distance analysis showed the G-group to be more polymorphic than the N-group, and closer to the Alaskan accessions. The N-group was highly homogeneous and this could also be seen in Fig. 1 where a number of N-specific AFLP fragments are present in all samples in the group, but G-specific fragments are not. The fragments in general were more variable in
the G-group, and a number of variable fragments in the G-group do not show up in the profiles of any N-plants.

It should be noted that the present genetic distance analysis (Fig. 2) has not placed the individual G- and N-samples with its population IS1, and this awaits further study for the reason for this to be determined. The pooled sample IS1 did not come from pooling the leaves or the DNA of the G- and N-plants, but was collected from a seed-derived accession maintained in a different location from the original introduction site.

The RFLP analysis of ribosomal genes (Fig. 3) supported the conclusions from the AFLP analysis in that: (i) the two lymegrass species were genetically differentiated; (ii) within-species polymorphism was higher in the Alaskan *L. mollis* than in the Icelandic *L. arenarius*; and (iii) within the Icelandic population of Alaskan *L. mollis* the N-plants were molecularly similar whereas the G-plants were polymorphic. The rDNA BamHI restriction profiles showed that *L. arenarius* had a common pattern consisting of three major bands (Fig. 3A: 11, 7 and 4 kb), whereas *L. mollis* had variable patterns (Fig. 3B,C: 9–10, 5–6, 4 kb and sometimes with an additional shorter fragment). As the ribosomal gene mapping (our unpublished results) revealed different chromosomal rDNA sites between these two *Leymus* species, the different restriction patterns of rDNA could well be explained by the genes having different genome origin. The individual plant analysis (Fig. 3C), which corresponded with the pooled sample analysis, showed the G-group to be variable between plants in their rDNA restriction patterns, especially at the second band position. In contrast, the N-plants were identical with respect to their rDNA restriction with this enzyme. Interestingly, some G-plants (e.g. G1, G3, G13) had two fragments at the second band position, indicating polymorphism between homologous sites as well.
Discussion

Species differentiation

Because AFLP profiles of lymegrass include many species-specific invariant bands, the technique is suitable as a check for taxonomy where similar phenotypes can lead to misclassification. Chromosome counting, and mapping of marker genes such as the ribosomal DNA (Ananthawat-Jónsson et al. 1997), can only confirm the species identity of a limited number of samples because both methods are tedious and time consuming. Other molecular and biochemical methods for the species identification are not as powerful. The RAPD method (random amplified polymorphic DNA) generates only a fraction of the resolution obtained from AFLP, as the profiles consist typically of about 10% of the number of scorable bands (our unpublished results). Electrophoretic analysis of seed endospermal protein has been used for species identification in lymegrass (Ahokas & Fredskild 1991; Ahokas 1992; Greipsson et al. 1997), but the method clearly cannot be used either on plants during their vegetative growth period, which is generally more than 3 years in perennial lymegrass, or in sterile wide hybrids. Karyotyping has demonstrated that such hybrids (i.e. hybrids between *Leymus arenarius* and *L. mollis*) occur in natural plant populations in Greenland where the two species coexist. Both interspecific (*Leymus × Leymus*) and intergeneric (*Leymus × Elymus*) hybrids have been documented in natural stands (Ahokas & Fredskild 1991). AFLP is probably the most effective means for accurate identification and verification of such hybrids, as the assay scans large areas of the genome in a single PCR. The method is especially suitable for selection of breeding material or for management of genetic resources which involves a large number of samples.

Fig. 2 AFLP neighbour-joining tree relating the two lymegrass species, *Leymus mollis* (Lm) and *L. arenarius* (La). The samples include populations (Lm-A, Lm-IS, La-86, La-87, La-00) and individuals (Lm-G, Lm-N). The numbers indicated on the main branches indicate discrimination between La (1) and Lm (2), where the Lm-group includes both the population (3) and individual samples (4). The sample La-86.14 is probably *L. mollis* (see text). The individual plant samples are separated into two groups consisting of the old (Lm-G) and the new (Lm-N) genotypes without overlap.

Fig. 3 Luminographs of ribosomal RFLP after Southern hybridization with BamHI-restricted genomic DNA. (A) Population samples of *Leymus arenarius* where three major fragments (11, 7 and 4 kb) are resolved. (B) Population samples of *L. mollis* (A61A and A204) and one individual plant sample (G1) showing polymorphism in the three fragment classes of 9–10, 5–6, 3–4 kb. (C) Individual plant samples of *L. mollis* (Lm-G, Lm-N) and one population sample (A106) showing polymorphism at the second fragment class among the G-plants, whereas the N-plants are identical in their rDNA–RFLP.
Polymorphism within species

The analyses of relatedness based on AFLP and RFLP of ribosomal genes show that the Icelandic populations of *L. arenarius* are more homogeneous than the Alaskan populations of *L. mollis* despite the similarity of both their latitudinal range (between 63° and 67°N) and their longitudinal span (4° to 6°) (with the exception of Alaskan population A204). The habitat is similar, as most lymegrass populations come from coastal areas less than 10 m above sea level. Furthermore, the more variable Alaskan population are closer together geographically (all but A204 are from Seward Peninsula and nearby areas) than those of the Icelandic lymegrass populations.

The observed differences in the extent of variation between the Alaskan *L. mollis* and the Icelandic *L. arenarius* may have been due to differences in the glacial history. *Leymus* is almost certainly a postglacial immigrant to Iceland (Björck *et al.* 1992; Hallsdóttir 1995) and may thus be poor in variation, but it may have existed for a long time in ice-free areas in Alaska and accumulated high levels of variation. *L. mollis* has been considered to consist of two subspecies (*mollis* and *villosissimus*) on the basis of a size difference of culms and spikes (Bowden 1957; Barkworth & Atkins 1984). Indeed, the occurrence of low taxonomic rank endemics, e.g. subspecies level, is one of the typical characteristics of glacial survival (Dahl 1987; Alm & Birks 1991), and this differentiation may have evolved in postglacial times, hence explaining the variation of Alaskan *L. mollis*.

The area where the present *L. mollis* samples originated from coincides with the overlap of the distribution of the two proposed subspecies. The ssp. *mollis* is abundant and widespread along both the Atlantic and Pacific coasts and some inland locations, while the smaller-stature ssp. *villosissimus* is rarer, and its distribution is primarily along Arctic coastlines and rivers. The two populations A061A (noted at the time of collection as resembling ssp. *villosissimus*) and A061B originate from collection points only a few metres apart, but their genetic distance from one another is greater than that between geographically distant populations. It is likely that the molecular polymorphism we have detected among the Alaskan *L. mollis* populations is a result of genetic differentiation at subspecies level as well as gene introgression between them.

In Iceland, *L. arenarius* is the only native lymegrass species with no indication of taxonomic differentiation within species. Thus gene flow among populations is probably not restricted by any genetic barriers, and consequently the populations would be expected to be relatively homogeneous. But as the species is primarily self-fertilized (Sigurbjörnsson 1960), the level of gene flow between populations may not have been very extensive, perhaps not more than what would account for the relatedness of adjacent populations. Land reclamation activities based on seed sowing in new areas will account for some of the relatedness, although most of the samples examined here are from old native sites. We therefore suggest that the present-day lymegrass populations in Iceland are more likely to have derived from a more widespread and continuous gene pool. Palaeovegetation studies based on pollen records of many dominant plant species have shown the vegetation to be much more abundant following the last deglaciation than it is at present (e.g. Hallsdóttir 1995). Birch, the most common tree species in Iceland, for example, had its peak of distribution about 10 000 years ago and our molecular studies (our unpublished results) show that the present-day polymorphisms are distributed continuously over the country, also suggesting a common gene pool.

Within-population homogeneity

Molecular polymorphism within a 40-years-old Alaskan *L. mollis* population in Southern Iceland (IS-1) has been examined on an individual plant basis. Both the whole genomic AFLP and the ribosomal RFLP show considerable variation among the old genotypes (G) whereas the new genotypes (N) are highly homogeneous. Some genotypes may have grown and propagated more vigorously than others, but this is not enough to explain such homogeneity over randomly collected samples. In addition there are new AFLP bands associated to the new genotypes (see for example Fig. 1). Rather than being the consequence of random genetic drift, these results lead us to suggest that stringent natural selection, together with mutation and perhaps sexual segregation, give rise to better-adapted genotypes. Although the old genotypes, probably Alaskan in origin, are still surviving, they are the newer ones that seem to be more aggressive in evading new and open areas and hence expanding its population leading to successful colonization in the Icelandic environment. Rapid evolutionary changes, adaptive or neutral, are known to have occurred in plants (Briggs & Walters 1997). Such changes can often be traced to changes in the environment, in the ecosystem, or after migration and colonization of a species in a new environment. An indication of rapid adaptation to specific habitat has been reported to have occurred with *L. arenarius* in Finland, where salt-tolerant types colonizing inland areas have become adapted to low salinity (Greipsson *et al.* 1997). The rapid molecular changes revealed in the present study may well be directly correlated to specific genes responsible for the physiological ability of the plants to survive and reproduce, or they may be the result of genomic turnover such as that due to activity of mobile elements under stress.

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This paper results from collaborative work between the Icelandic Agricultural Research Institute, the University of Iceland and the John Innes Centre in Norwich, UK. We investigate all aspects of lymegrass genetics, ecology and breeding, and in particular those involving population dynamics in order to explore the species potential in multiple uses including land reclamation, ecological succession in new environments, and in genetic resource management. We use plants in the field and create new hybrids and genotypes for studying gene introgression between closely related species and between lymegrass and crop species like wheat.